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Validation of an improved reversed-phase HPLC assay with reductive electrochemical detection for the determination of artemisinin derivatives in man
Validation of an Improved Reversed-Phase High-Performance Liquid Chromatography Assay With Reductive Electrochemical Detection for the Determination of Artemisinin Derivatives in Man

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Summary: For the determination of artemisinin (ART) and analogs, a reversed-phase high-performance liquid chromatography method using reductive electrochemical detection (ED) was set up with some important modifications as compared to previously published assays. A different technique of deoxygenating resulted in a factor 2–3 lower background current. A Spectroflow 400 liquid chromatograph in combination with a Triathlon autoinjector coupled to a Decade electrochemical detector was used. The detector was operated in the reductive mode as a closed system under chromatography grade helium to exclude any access of oxygen. The Decade has a glassy carbon electrode and a reference Ag/AgCl electrode. Infrequent electropolishing was required implicating a very stable system. By increasing acetonitril or lowering the pH of the
mobile phase, the various derivatives could be determined in the same chromatogram. The assay was validated using artemether (ATM) and dihydroartemisinin (DHA) as test substances. In the concentration range seen in people after usual doses (5 to 220 ng/ml), the assay performs with adequate accuracy and precision. The interassay and intraassay precision are <6% for ATM. For DHA, the interassay and intraassay precision are <9%. The accuracy expressed as the deviation from the expected concentration varies from −1% to +4.5% for the intraassay ATM-determinations and from +1% to +6.3% for the interassay measurements. For DHA, the accuracy is somewhat less, varying from −0.3% to −9.5% for the intraassay measurements and −0.6% to +2.6% for the interassay measurements. The reproducibility of the assay, measured over a time period of 3 months, is good for ATM and DHA with an interassay precision of <18% in 70 repetitive samples and an accuracy varying from −0.6% to +7.6%. In a cross-check with two other reference laboratories who used comparable methods of determination, a strong correlation (correlation coefficient > 0.98) was achieved. The method was applied in a study in which artemether was administered orally to healthy white subjects. We consider high-performance liquid chromatography with electrochemical detection an accurate and precise method for quantitative determination of artemisinin derivatives in pharmacokinetic studies. **Key Words:** Artemisinin derivative—High-performance liquid chromatography—Electrochemical detection—Validation—Artemether—Dihydroartemisinin—Assay.

_Qinghaosu_ or artemisinin (ART) is isolated from the herb _Artemisia annua_. It is the parent compound of a new class of antimalarials with great potential value. Since 341 A.D., this herb has been used for fever, hemorrhoids, and 32 other diseases in traditional Chinese medicine (1). In 1974, it was found to possess antiplasmodial activity and to be effective in the treatment of malaria. Emerging resistance and problematic side-effects for known antimalarial drugs have stimulated the search for new therapies. Semisynthetic derivatives such as artemether
M. A. VAN AGTMAEL ET AL

(ATEM), arteether (ATE), and artesunate (ATS) have been developed with sometimes greater antimalarial activity and permit parenteral administration (2). Figure 1 shows the chemical structure of artemisinin and some derivatives. It is a sesquiterpene lactone with a characteristic endoperoxide bridge essential for its antimalarial activity (3). During a 1992 World Health Organization conference, it was emphasized that efforts should be made to develop robust analytical methods for monitoring pharmacologic and idiosyncratic toxicity in patients treated with these analogues (4). The development of selective analytical methods for the determination of artemisinin and its analogues and metabolites in biologic fluids poses challenging problems. All are thermally labile, lack ultraviolet (u.v.) absorption or fluorescent chromophores, and do not possess functional groups with potential for derivatization. In this article, we describe high-performance liquid chromatography (HPLC) with reductive electrochemical detection (HPLC-ED), which is regarded as more sensitive than HPLC-UV and the specific reduction of the peroxide bridge gives this method a higher specificity than radioimmunoassay (RIA) or bioassay (5,6). High-performance liquid chromatography with reductive electrochemical detection uses the endoperoxide bridge of the active artemisinin derivatives. This structure is reduced at the working-

MATERIALS AND METHODS

Materials

Stock solutions containing 100 μg/ml dihydroartemisinin (DHA), 100 μg/ml artemisinin (ART), 100 μg/ml arteether (ATE), and 100 μg/ml artemether (ATM) in 100% ethanol were prepared. Artemisinin, DHA, and
electrode, and the signal of the resulting electric current is used for detection. Inherent difficulties of the method are that it requires rigorous deoxygenation of samples and mobile phase and an electrochemical detector that is expensive and difficult to operate. However, the need for special laboratory facilities and highly trained and experienced technicians makes the assay available in only a limited number of laboratories.

![Chemical structure of artemisinin and some derivatives.](image)

- R = \=O \text{Artemisinin}
- \text{-OH} \text{Dihydroartemisinin}
- \text{-OCH3} \text{Artemether}
- \text{-OC2H5} \text{Arteether}
- \text{-OCO-(CH2)2CO2H} \text{Artesunate}

**FIG. 1.** Chemical structure of artemisinin and some derivatives.

ATE were obtained from ACF Farma (Maarssen, The Netherlands), whereas ATM was made available by Novartis (Basle, Switzerland). For daily work, the stock-solutions were further diluted to 1 μg/ml in water:ethanol (1:1). All chemicals and solvents used in our assay procedure are of analytical/chromatographic grade. Acetonitril, ethanol, 1 M natrium hydroxide, 100 mM acetate buffer, pH 5.0, ethyl acetate, 1-chlorbutane, toluene, and dichlorodimethylsilane are obtained from Merck (Darmstadt, Germany).

**Chromatography**

The analytical equipment was set up as previously published by Melendez et al (5) with some rather important modifications. Instead of the BAS 200 liquid chromatograph (Bioanalytical Systems, West Lafayette, IN, U.S.A.), a Pump spectroflow 400 (Applied Biosystems B.V., Rotterdam, The Netherlands) (flow rate, 1.5 ml/minute) liquid chromatograph with Triathlon autoinjector (Spark Holland Separations, Hendrik Ido Ambacht, The Netherlands) attached to an electrochemical detector (Decade; Antec, Leiden, The Netherlands) were used. The instrument is operated in the reductive mode as a system with stainless steel tubings, well air-closed to exclude any oxygen at the detector under chromatography grade helium. The electrochemical detector contains...
ASSAY OF ARTEMISININ DERIVATIVES WITH HPLC AND REDUCTIVE ED

glassy carbon working electrodes, a reference Ag/AgCl, and an auxiliary 316 stainless steel electrode and has a working potential of −1 V. The electrode was wiped when the mobile phase was changed, and electropolishing was conducted once a year, implicating a very stable system. The Triathlon autoinjector (Spark Holland Separations) was used in the “user-mode;” it executed sample degassing with argon for 15 minutes and injection of the deoxygenated sample with a 100 μl loop. An inner and outer needle perforated the membrane of the vial with extracted sample. Rigorous deoxygenating took place by blowing argon in the sample from the inner needle located under the fluid surface. The outer needle removed all the air above the sample. After 15 minutes, aspiration of the degassed sample took place through the inner needle into a syringe while the outer needle blew argon into the vial. The syringe loaded the 100-μl loop from which the sample was injected into the system. Chromatographic separations were obtained with a Versapack column (Alltech, The Netherlands): CN-column, 300 × 4.6 mm, 10 μm particle size, with cyanopropyl as stationary phase, maintained at 30°C. The composition of the mobile phase consists of 60% acetate buffer (100 mM) and 40% acetonitril, and has a pH of 5. The mobile phase was thoroughly deoxygenated for at least 24 hours before sample injection. During the process of recirculating the mobile phase, the whole system will then become deoxygenated. Renewal of the mobile phase at
least every 3 to 4 weeks is needed because after this time the acetonitril concentration decreases and the retention time will prolong. With minor changes in the mobile phase, such as increasing acetonitril for the much less polar arteether or lowering the pH for the pH sensitive artemisunate, these compounds can be determined in the same chromatogram together with dihydroartemisinin, artemisinin, and artemether (Fig. 2). The chromatograms are recorded and analyzed with Kontron Datjet integrator software (Kontron Instruments S.p.A., Milano, Italy).

Sample Extraction

All glass materials are silanized to minimize drug adsorption to the glass. We usually use 1 ml of serum, although smaller sample sizes can be processed. Before extraction, 25 μl of the internal standard solution (1 μg/ml ART for the assay of the derivatives and 1 μg/ml ATM for the determination of ART) is added to the samples, followed by vortex mixing for 20 seconds. Then 10 ml 1-chlorobutane:ethyl acetate (9:1) is added and shaken for 10 minutes on a mechanical shaker. The samples are centrifuged for 10 minutes at 4000 rpm at a temperature of 23°C. The organic layer is put into clean glass tubes and evaporated under a continuous nitrogen stream at room temperature. The samples are dissolved in 250 to 500 μl ethanol:water (1:1) and injected after deoxygenation.

VALIDATION RESULTS

As shown in a chromatogram of high concentration standard solutions in ethanol in Figure 2, with our reversed-phase system retention times in the same chromatogram for dihydro-artemisinin, artemisinin, arteether, and arteether are 5.1, 9.2, 10.8, and 16.1 minutes, respectively. Figure 2A is a plasma sample spiked with 50 ng/ml ATM and 50 ng/ml DHA and shows an adequate baseline separation without interferences from excipients. Peak B and C in the chromatogram are the α- and β-DHA. It is unknown, and which peak represents which anomer remains unclear in the literature probably...
because the pure substance of the anomer is not available. The α-configuration may be the preferred conformation and the more polar compound, and therefore, comes first in the chromatogram. In our determination we have always used the first and largest peak for the DHA-concentration. Summation of the peaks generally is not done and is not advisable because we are not informed about the electrochemical behavior of the two anomers. Earlier experiments (5) have described an equilibration period of 3 to 4 hours, and this was approximately the time period the samples were left to stand before injection.

Recovery

We measured the recovery of the extraction procedure by comparing the peak height, which was acquired from measurement of a spiked plasma sample with known concentration, with the peak height from measurement of a stock solution with the same concentration, which was directly injected into the HPLC system. The recovery of the four compounds approached 100%.

Calibration

Solutions of ATM and DHA in ethanol:water (50:50), least 5 ng/ml for ATM in human serum (interassay precision 6%, accuracy +2.2%) (Table 1). According to these standards DHA had a similar limit of quantification of 5 ng/ml (interassay precision 9%, accuracy +2.2%) (Table 2).

Assay Precision and Accuracy

Intraassay Precision and Accuracy

We analyzed four spiked human serum samples with concentrations ranging from 10 to 220 ng/ml six times on the same day to test intraassay precision or within-day variability. The results for ATM are shown in Table 1 and for DHA in Table 2. The coefficient of variation ranged from 1.9% to 6% for ATM and 3.6% to 8.5% for DHA. As a measure of accuracy, the deviation from expected concentration of the mean values ranged from −1.0% to +4.5% for ATM and −0.3% to −9.5% for DHA.

Interassay Precision and Accuracy

The interassay precision or day-to-day variability was estimated by analyzing four spiked human serum samples with concentrations ranging from 5 to 220 ng/ml ATM (Table 1) and DHA (Table 2) on 5 different days. The coefficient of variation ranged from 3.49% to 5.88%
ranging from 0 to 200 ng/ml, were injected in the HPLC-ED apparatus to assess detector linearity. Peak height was plotted against the amount of compound injected. In this range linearity was confirmed ($r > 0.99$). Calibration curves were obtained by spiking drug-free plasma samples with standard solutions to produce concentrations of 0, 10, 50, 100, 200 ng/ml of ATM and DHA. Artemisinin as internal standard was also added. The samples were taken through the extraction and assay procedure and the peak heights were plotted against the corresponding drug concentrations. Linear regression analysis yielded mean ($n = 11$) values for slope and intercept for the equation of the calibration plots for ATM of $y = 0.0183x + 0.0170$ and for DHA $y = 0.0140x + 0.0727$ in which $y$ is the peak height ratio of ATM or DHA to the internal standard (no units) and $x$ is the concentration of the calibration samples in ng/ml. The mean values for the correlation coefficients were $r = 0.998$ for DHA and $r = 0.998$ for ATM.

**Limit of Quantitation**

The limit of quantitation (LOQ), defined by the lowest amount quantitatively determined in a sample volume of 1 ml with a precision around the mean value in 5 replicates within 15% and an accuracy within 20% was at for ATM and 2.62% to 9.00% for DHA. The deviation from the expected concentration ranged from $+0.95\%$ to $+6.34\%$ for ATM and $-2.46\%$ to $+2.56\%$ for DHA.

**Quality Control Samples (Method Validation During Study)**

Four freshly prepared, spiked quality control samples in two concentrations (50 and 200 ng/ml) for ATM and

| TABLE 1. Intraassay and interassay precision and accuracy of artemether determinations |
|-----------------------------------|-------------------|-------------------|-------------------|
| Expected concentration | Intra-assay mean conc. ± SD | Precision (CV %) | Deviation from expected % |
| ATM 220 | 220.8 ± 4.1 | 0.19 | +0.4 |
| DHA 50 | 52.2 ± 3.1 | 0.59 | +4.5 |
| ATM 25 | 24.8 ± 1.5 | 0.60 | -1.0 |
| DHA 10 | 10.3 ± 0.6 | 0.54 | +3.0 |
| DHA 5 | 10.6 ± 0.4 | 0.35 | +6.3 |

Conc., serum concentration in ng/ml; SD, standard deviation; CV, coefficient of variation = SD* 100/mean.

Deviation from expected = (mean conc. - expected conc.)* 100/expected conc.
DHA were measured during 70 replicate assays within-run time over a 3-month period. We used stock solutions different from those used for the calibration curves. The spiked samples were stored under the same conditions as the study samples. For ATM, the coefficient of variation was <18% in the low and high range concentration. Deviation from expected concentration varied from -0.6% to +7.6%. For DHA the coefficient of variation was <12% with a deviation from expected of -5.1% to +0.2% (Table 3). As many factors can influence the stability of HPLC-ED, such as temperature and pH, the interassay precision can be rather high and thus will be higher than

When reviewing the original chromatograms it was apparent that these outlying values were produced by the duplicates measured during the second run. These chromatograms had a rather unstable baseline, a known problem with electrochemical detection in the reductive mode, probably caused by insufficient degassing of the samples. If we omitted these defective chromatograms and, for comparisons, only used the values of duplicates that could be trusted, the correlation coefficient between added and found values further increased to 0.998 and the mean difference (±SD) between given and found decreased to 6.1 ± 6.2 ng/ml, which is <10% of the mean added artemether concentration. For DHA an in-house validation was performed by blinding and randomizing 10 samples with known DHA concentrations ranging from 0 to 200 ng/ml. Eight of ten measured samples deviated from 0% to 18% from the expected concentrations. One sample was 23% lower than the actual concentration and one was an obvious outlier. The mean absolute difference was 9.8 ng/ml, 11.7% of the mean added DHA-concentration.

**In Vivo Cross Check**

With samples from a study in healthy human subjects who had received a single oral dose of 80 mg artemether, a comparison was done between our assay (AMC) and

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**TABLE 2.** Intraassay and interassay precision and accuracy of dihydroartemisinin determinations

<table>
<thead>
<tr>
<th>Expected concentration</th>
<th>Intra-resp.inter assay</th>
<th>Measured mean conc. ±SD</th>
<th>Precision (C V %)</th>
<th>Deviation from expected %</th>
</tr>
</thead>
<tbody>
<tr>
<td>220</td>
<td>Intra</td>
<td>205.7 ± 8.6</td>
<td>4.2</td>
<td>-6.5</td>
</tr>
<tr>
<td></td>
<td>Inter</td>
<td>218.8 ± 5.7</td>
<td>2.6</td>
<td>-0.6</td>
</tr>
<tr>
<td>50</td>
<td>Intra</td>
<td>45.2 ± 1.6</td>
<td>3.6</td>
<td>-9.3</td>
</tr>
<tr>
<td></td>
<td>Inter</td>
<td>51.3 ± 3.4</td>
<td>6.6</td>
<td>+2.6</td>
</tr>
<tr>
<td>25</td>
<td>Intra</td>
<td>24.9 ± 2.1</td>
<td>8.5</td>
<td>-0.3</td>
</tr>
<tr>
<td></td>
<td>Inter</td>
<td>9.7 ± 5.0</td>
<td>5.0</td>
<td>-2.7</td>
</tr>
<tr>
<td>10</td>
<td>Intra</td>
<td>9.8 ± 0.6</td>
<td>5.7</td>
<td>-2.5</td>
</tr>
<tr>
<td></td>
<td>Inter</td>
<td>5.1 ± 0.5</td>
<td>9.0</td>
<td>+2.2</td>
</tr>
<tr>
<td>5</td>
<td>Intra</td>
<td>9.8 ± 0.6</td>
<td>5.7</td>
<td>-2.5</td>
</tr>
<tr>
<td></td>
<td>Inter</td>
<td>5.1 ± 0.5</td>
<td>9.0</td>
<td>+2.2</td>
</tr>
</tbody>
</table>
the intraassay precision.

**Measurement of Unknown Samples**

Ten blinded samples with known concentrations of ATM, prepared by Novartis Pharma AG (Basel, Switzerland) and ranging from 0 to 193.5 ng/ml were analyzed in our laboratory in duplicate. On average 109.4 ± 18.9% (mean ± SD) of the added artemether was found by our laboratory in the samples. The absolute mean difference was 9.5 ng/ml. However, when the given values were plotted versus the found estimates it became obvious that the highest found values (samples 4 and 8) were outlying observations. Without these two values, the correlation coefficient increased from 0.970 to 0.996.

**Artemether Pharmacokinetics in Healthy Subjects**

Figure 5 shows the concentration time profiles of ATM and DHA. The concentrations were measured with our assay and modeled with a one-compartment open

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**TABLE 3. Quality control samples measured in a time period of 3 months**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Samples (n)</th>
<th>Added conc. (ng/ml)</th>
<th>Measured conc. (ng/ml) (mean ± SD)</th>
<th>Coefficient of variation (%)</th>
<th>Deviation from theory (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemether</td>
<td>70</td>
<td>50</td>
<td>53.8 ± 7.5</td>
<td>13.9</td>
<td>+7.6</td>
</tr>
<tr>
<td></td>
<td>68*</td>
<td>200</td>
<td>198.8 ± 35.0</td>
<td>17.7</td>
<td>-0.6</td>
</tr>
<tr>
<td>Dihydro-artemisinin</td>
<td>70</td>
<td>50</td>
<td>50.1 ± 5.1</td>
<td>10.2</td>
<td>+0.2</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>200</td>
<td>189.8 ± 22.1</td>
<td>11.7</td>
<td>-5.1</td>
</tr>
</tbody>
</table>

* Two undetermined samples because of disturbances in the high-performance liquid chromatography chromatogram.

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Ther Drug Monit. Vol. 20. No 1, 1998
model with a lagtime for ATM and a biotransformation
time for DHA. These results were obtained in a represen­tative individual during a kinetic study in which single oral doses of 100 mg artemether were given to seven healthy white subjects. PK-parameters of ATM in these subjects reveal a mean elimination half-life ($t_{1/2}$) of 1.3 hours, a mean maximum concentration ($C_{\text{max}}$) of 29 ng/ml, and for DHA, a mean $t_{1/2}$ of 1.4 hours and a mean $C_{\text{max}}$ of 32 ng/ml was found.

Hydroxylated metabolites of artemisinin derivatives are likely to have retention times different from our analytes. For these reasons we find no Qinghaosu-metabolites interfering in the chromatogram.

The two important chemical groups to which drugs belong, which are reducible and possibly interfering with the quantitation, are quinones and nitro-compounds. The first group mainly consists of anthraquinone-containing contact-laxatives, such as senna and cascara, which are
Specificity of the Assay

Only Qinghaosu-metabolites with the intact peroxide bridge, such as DHA, are detectable with ED. Artesunate, artemether, and arteether are transformed into dihydroartemisinin, which is subsequently converted into inactive metabolites that have lost their peroxide moiety.

We studied flunitrazepam and nitrazepam, which belong to the nitro compounds and found no interference with the electrochemical detection of artemisinin-derivatives.

In plasma samples from healthy volunteers and Asian, white, and black patients with malaria, we never experienced interfering peaks, which supports the specificity of the method.

**FIG. 4.** Dihydroartemisinin in vivo-cross check. AMC, Academic Medical Center Laboratory Clinical Pharmacology; Ref-lab, Reference Laboratory.
DISCUSSION

Until recently artemisinin and its related compounds were very difficult to assay in body fluids (11). Adequate sensitivity and acceptable reproducibility of reversed-phase HPLC with reductive electrochemical detection for the determination of ATM and DHA in human plasma has been described earlier in the literature (5,6,9,10). However, we felt that in view of the many potential intra-assay precision) or under different conditions (from day-to-day or interassay precision) always <6%. For DHA the interassay and intraassay precision is <9%. The accuracy of the ATM assay expressed as the deviation from the expected concentration varies from -1% to 6.4%. For DHA, the accuracy is somewhat less varying, from -9.5 to +2.6%.

The precision over time is good for ATM and DHA with an interassay precision of <18% in 70 repetitive samples measured in a 3-month period. The accuracy varied from -0.6% to +7.6%.

However one has to be cautious when referring to accuracy of DHA estimates. DHA is present in the sample in an unknown ratio of the two dihydroartemisinin anomers. The influence on this ratio of different solvents and various conditions such as pH, temperature and, most importantly, differences in electrochemical behavior between these two stereoisomers, are mostly unknown variables. The stereoisomers of DHA are two different compounds with different physical properties, e.g., showing different retention times because of different polarity. It is possible that this may result in a different response with the ED. In fact, artemisinin and ATM show different responses despite having relatively similar structures with regard to the endoperoxide moiety. A proper interpretation of DHA chromatographic
tial and encountered difficulties, this methodology could only be used on a routine basis after the assay was rigorously validated. During the validation procedures we used ATM and DHA as test substances. For validation purposes our assay was subjected to three blinded cross checks, two with ten samples spiked with either ATM or DHA and one with two other laboratories where the same ten samples after in vivo drug administration were assayed. In Figure 2 it can be seen that at least four different derivatives can be separated in the same chromatogram. Artemether is eluted much earlier than arteether, indicating a considerable difference in polarity, which could have important kinetic implications. Our internal standard assays for the various artemisinin analogs went through a within-laboratory validation procedure for concentrations ranging from 5 to 220 ng/ml, which showed an overall precision of 9% and accuracy of 9.5%. Calibration curves with high correlation coefficients show the adequate linearity of the test procedure, which indicates that the test results are directly proportional to the concentration of analyte in the sample. The low intercept parameter argues against interfering peaks. A limit of quantification in the 5 to 10 ng/ml range was achieved for ATM and DHA. The precision of the arte- mether determination expressed as coefficient of variation is under the same conditions (within one-day or results is only possible if this information is available. In our opinion these problems are seriously underestimated in the literature. Therefore one can define the precision of DHA estimations but hardly the accuracy.

Compared to the assays of two other laboratories, our assay was further evaluated. Concordance between the results for concentrations higher than the detection limits of our laboratory and the other laboratories that used the same equipment and procedures was displayed by correlation coefficients ranging from 0.983 to 0.998. Finally, the method was used in a study in healthy subjects providing pharmacokinetic results that were reproducible and matching with similar methods. This illustrates that the assay can be applied in clinical pharmacokinetic studies. Drawbacks of the method are the necessary thorough deoxygenation of the samples and the mobile phase and an electrochemical detector that is expensive and difficult to operate.

The use of artemisinin and derivatives for the treatment of malaria is already extensive but will further rise in the near future (12,13). Pharmacokinetic studies are necessary to detect interindividual differences, to understand concentration-effect relationships in this new group of drugs and to predict optimal dosing regimens (14). Recurrent problems with the equipment can slow progress with the determinations. However, this new
technique has enabled us to conduct kinetic studies with these very interesting antimalarials. Reports of studies in which we applied our methodologies to questions concerning the pharmacokinetics of Qinghaosu-derived compounds, both in healthy subjects and patients with falciparum malaria have been published (4,15–17), and from this experience we consider HPLC-ED an appropriate determination technique to estimate concentrations of artemisinin derivatives for the study of the pharmacokinetics of these compounds in man.

REFERENCES


