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RAPID HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF ACETAMINOPHEN IN SERUM AND TISSUE HOMOGENATES

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SUMMARY

The quantitation of a hepatorenal toxic drug, acetaminophen, in blood and target organ tissues is needed for toxicokinetic and distribution studies. A rapid, sensitive and simple method is described to assay acetaminophen in rat serum and liver or kidney homogenates by reversed-phase high-performance liquid chromatography, using an octadecyl (3 μm particle size) Apex column, a mobile phase consisting of a mixture of distilled water—acetonitrile (86:14) and ultraviolet detection at 245 nm. Short retention times of ca. 3.75 and 6.25 min are observed for acetaminophen and the internal standard (sulfamerazine), respectively. A sensitivity of 50 ng/ml is easily achieved for 100-μl serum and liver or kidney homogenate samples. The proposed method proved to have satisfactory recovery, precision and accuracy. The preliminary results obtained with human plasma of volunteers and of patients treated with various drugs show that the assay, with a sensitivity of 25 ng/ml, would be of considerable interest in clinical monitoring of acetaminophen.

INTRODUCTION

Acetaminophen is an extensively used over-the-counter drug which has hepatorenotoxic properties in many species including man [1]. In order to conduct pharmacokinetic and target organ distribution studies in small animals

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such as the rat, a very sensitive method requiring small volumes of samples is needed.

Many methods for the determination of acetaminophen in serum or plasma have been published, most of them using reversed-phase high-performance liquid chromatography (HPLC) and UV detection around 254 nm. Sensitivity levels of 200 ng/ml or less have been reached with electrochemical detection [2], radiolabeling [3, 4] or simple UV detection [5—10]. A very few methods describe determination of acetaminophen from tissue homogenates [4, 11], and lack of sensitivity [11] or use of [^{14}C]acetaminophen [4] decrease their applicability. Here we describe a rapid and very simple assay for acetaminophen in serum and liver or kidney homogenates requiring only 100 μl of a biological sample and reaching a 50 ng/ml level of sensitivity with UV detection at 245 nm.

EXPERIMENTAL

Materials

Acetaminophen was obtained from Sigma (St. Louis, MO, U.S.A.). Sulfamerazine, the internal standard, was also purchased from Sigma. Isopropanol and acetonitrile, HPLC-grade (LiChrosolv) were obtained from BDH (Toronto, Canada).

Apparatus

A Varian Model 5010 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with a VariChrom variable-wavelength UV—VIS detector and a Varian G 2500 recorder was used. Separations were performed on a 15 cm × 4.5 mm I.D. octadecyl (3 μm particle size) Apex column (Jones Chromatography, Columbus, OH, U.S.A.). A Rheodyne® 1-μm filter (Rheodyne, Cotati, CA, U.S.A.) was installed between the Valco loop injector (Varian) of the chromatograph and the analytical column.

Sample preparation and liquid chromatographic procedure

Serum was obtained from centrifuged blood (500 *g* for 10 min) collected from male Sprague—Dawley rats (Charles River, St. Constant, Canada) which were then sacrificed for removal of liver and kidneys. The rat organs were homogenized in 1.15% potassium chloride with a Brinkmann Polytron apparatus (Brinkmann Instruments, Westbury, NY, U.S.A.). The homogenates contained 250 mg of organ tissue per ml. Aliquots of 100 μl of either serum, liver or kidney homogenate were pipetted into 15-ml extraction tubes. A 1-ml mixture of acetonitrile—isopropanol (50:50) containing 8 μg/ml sulfamerazine, the internal standard, was then added to each tube as the extraction solvent. The tubes were then vortex-mixed for 30 s and centrifuged at 500 *g* for 2 min to precipitate proteins. A 600-μl volume of the clear supernatant was then transferred to conical glass tubes and evaporated at 40°C under vacuum with a Buchler vortex evaporator (Buchler Instruments, Fort Lee, NJ, U.S.A.). Each residue obtained was dissolved in 300 μl of distilled water and 50 μl were then injected into the reversed-phase column at ambient temperature.

The mobile phase consisted of a mixture of distilled water—acetonitrile

(86:14). The flow-rate was 1 ml/min and the eluate was monitored at 245 nm. Detector sensitivity was set between 0.1 and 0.5 a.u.f.s. Chart speed of the recorder was fixed at 0.254 cm/min.

RESULTS AND DISCUSSION

Retention times and background

Fig. 1 shows representative chromatograms (I and II) from a rat serum extract containing or not acetaminophen and the internal standard, sulfamerazine. Retention times for acetaminophen and sulfamerazine are approx. 3.75 and 6.25 min, respectively. Total time of analysis is less than 7.5 min.

Analysis of several extracts of xenobiotic-free samples of rat serum and liver or kidney homogenates has demonstrated that endogenous serum or tissue homogenate constituents do not interfere with peaks corresponding to acetaminophen or sulfamerazine, as illustrated in Figs. 1 and 2 for serum and liver homogenate, respectively, for example.

Recovery

The recovery of the extraction was evaluated by spiking blank serum and organ homogenate samples with known amounts of acetaminophen and by comparing the peak heights obtained after extraction with those from standard aqueous solutions. Analytical recovery was found to be complete

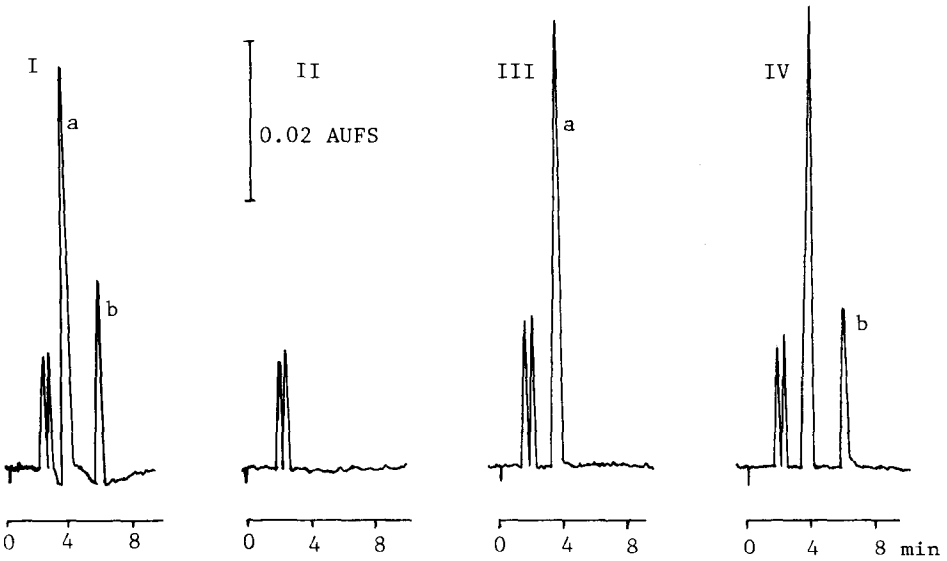

Fig. 1. Chromatograms of (I) extracted spiked rat serum sample containing acetaminophen at a concentration of 50 μg/ml and sulfamerazine at an equivalent concentration of 80 μg/ml; (II) extracted acetaminophen- and sulfamerazine-free rat serum sample; (III) an extract of serum sample obtained from a rat after the intraperitoneal administration of a single dose of acetaminophen (750 mg/kg); (IV) an extract of the preceding serum sample (chromatogram III) containing the internal standard. Peaks: a = acetaminophen; b = sulfamerazine.

for all media at two different concentrations, namely 200 and 0.5 μg/ml of serum or tissue homogenate. The recoveries found at these concentrations were 101.4% (S.E. = 3.2%, n = 5) and 105.3% (S.E. = 1.6%, n = 5), respectively.

Smaller volumes of serum or homogenate were also tested, down to 15 μl. Distilled water was used to complete the volume to 100 μl. Recoveries obtained with these lower volumes of samples were all 100% with minimal variation [15, 20, 40 and 50 μl of sample at 0.5 and 200 μg/ml gave an overall mean recovery value of 102.6% (S.E. = 1.8%, n = 20)]. Sulfamerazine, the internal standard, gave a recovery of 102.1% (S.E. = 1.9%, n = 5), independent of the extracted medium. Analysis of variance revealed that there was no significant difference in recoveries for the different sample volumes and compound (acetaminophen and sulfamerazine) concentrations.

Sensitivity and calibration curve

Acetaminophen was added to drug-free sera and tissue homogenates in amounts equivalent to 0.05—200 μg/ml of serum or 0.20–800 μg/g of tissue, respectively. The first concentration, which is included in the calibration curve, is the sensitivity limit, which has been determined from a detector signal-to-noise ratio of 3. Limit of detection is 0.03 μg/ml for serum and 0.12 μg/g for organ tissue with a signal-to-noise ratio of 2. Acetaminophen concentrations are quantitated by calculating the ratio of the peak height of the drug to the peak height of the internal standard. The relation of this ratio to serum or tissue acetaminophen concentration is linear with a correlation coefficient, r, of 0.999. The equations of the calibration curves for the different media are given in Table I.

TABLE I

LINEAR REGRESSION ANALYSIS OF STANDARD CURVES

Sample	Equation of straight line for eight standard samples*
Serum	$y = (0.0250 \pm 0.0030)x + (0.1850 \pm 0.0110)$
Liver	$y = (0.0062 \pm 0.0005)x + (0.1950 \pm 0.0200)$
Kidney	$y = (0.0065 \pm 0.0005)x + (0.1900 \pm 0.0150)$

*The values of the slope and y-intercept are the average value ± standard error (n = 5); y = peak-height ratio; x = concentration of acetaminophen in μg/ml of serum or μg/g of organ tissue.

Precision and accuracy

Both the within-day and the between-day precision were evaluated by spiking sera and tissue homogenates with three different concentrations of acetaminophen, and by calculating the coefficient of variation with ten or nine measurements, respectively; the results are presented in Table II. The low values of the coefficients of variation indicate that the procedure is highly reproducible.

Accuracy was evaluated by spiking sera and tissue homogenates with five different concentrations not included in the calibration curves and by measuring the variations between the theoretical concentration and the one

TABLE II

WITHIN-DAY AND BETWEEN-DAY PRECISION FOR THE DETERMINATION OF ACETAMINOPHEN CONCENTRATION IN RAT SERUM AND LIVER OR KIDNEY HOMOGENATES

Concentration in sample	Coefficient of variation* (%)	
	Within-day (n = 10)	Between-day (n = 9)
Serum (μg/ml)		
200	2.5	5.1
50	3.2	4.6
2.5	3.9	6.5
Liver (μg/g)		
800	1.7	3.2
200	3.6	4.0
10	2.5	5.1
Kidney (μg/g)		
800	2.6	2.5
200	4.0	3.1
10	5.1	6.5

*Coefficient of variation of peak-height ratios.

TABLE III

ACCURACY IN THE DETERMINATION OF ACETAMINOPHEN CONCENTRATION IN RAT SERUM AND LIVER OR KIDNEY HOMOGENATES

Theoretical concentration (μg/ml)			Deviation (%)		
Serum	Liver	Kidney	Serum	Liver	Kidney
150.0	600.0	600.0	8.0	7.8	7.6
75.0	300.0	300.0	6.4	5.5	5.8
37.5	150.0	150.0	3.3	4.5	5.3
3.75	15.0	15.0	10.6	8.9	8.0
0.5	2.0	2.0	11.3	10.5	9.9
Mean			7.92	7.44	7.32
Standard error			1.45	0.98	0.83

obtained by assaying the spiked samples. Results, presented in Table III, show very low deviations.

Interference and application

A series of commonly used drugs were assayed with the chromatographic conditions used for acetaminophen in order to evaluate a possible interference of these substances. The list of these products and their respective retention times is given in Table IV. None of them was found to interfere significantly with the peaks of acetaminophen or sulfamerazine.

TABLE IV

RETENTION TIMES FOR SOME DRUGS

Drug	Retention time (min)	Drug	Retention time (min)
Amobarbital	2.5	Salicylic acid	3.0
Caffeine	4.4	Sulfathiazole	3.1
Carbamazepine	2.5	Theophylline	2.5
Codeine phosphate	2.6	Theophylline ethylenediamine	3.2
Phenobarbital	3.1	Vitamin C	1.9
Quinidine	5.6		

TABLE V

ANALYSIS OF SERUM SAMPLES FROM PATIENTS OR VOLUNTEERS HAVING RECEIVED DIFFERENT DRUGS

Drug given	Peak retention time[a] (min)	n[b]	Drug serum concentration[c] (μg/ml)
Carbamazepine[d]	2.24	2	4.7 and 7.1
Cardizem[e]	None	1	—[f]
Ibuprofen[g]	None	1	—[h]
Phenobarbital[d]	3.20	1	12.6
Phenytoin[d]	2.62	1	5.4
Theophylline[d]	2.24	8	4.6—19.5
Valproic acid[d] and carbamazepine	2.24	1	4.3 7.7

[a]Peak retention times observed when serum samples were assayed according to the HPLC method described above.
[b]Number of serum samples obtained from different volunteers or patients.
[c]Drug serum concentration as measured by Dr. Claire Dupuis who supplied the serum samples.
[d]Serum samples kindly supplied by Dr. Claire Dupuis of the Hôpital Ste. Justine in Montreal.
[e]Serum sample kindly supplied by Dr. Gilles Cayer of the Université de Montréal.
[f]High concentration in an intoxication case.
[g]Serum sample kindly supplied by Dr. Jean-Guy Besner of the Université de Montréal.
[h]Unknown concentration following a therapeutic dose of 300 mg.

In order to evaluate the interaction of both parent drug and metabolites, serum samples of patients having received a variety of drugs were obtained and analyzed according to the method described above. None of these serum samples studied were found to give an interference peak with the one of acetaminophen or sulfamerazine (Table V).

We tested also the possible interference with the more polar metabolites of acetaminophen which can be found in relatively small amounts in serum and in higher concentrations in the liver [4]. The major end products of acetaminophen are the sulfate, the glucuronide and the mercapturate conjugates which have retention times of 2.1, 2.0 and 2.0 min, respectively. Presence of the acetaminophen metabolites in serum is particularly of concern in the cases of accidental ingestion of high doses in man and of administration of high toxic doses in laboratory animals. Of all the techniques found in the literature, none

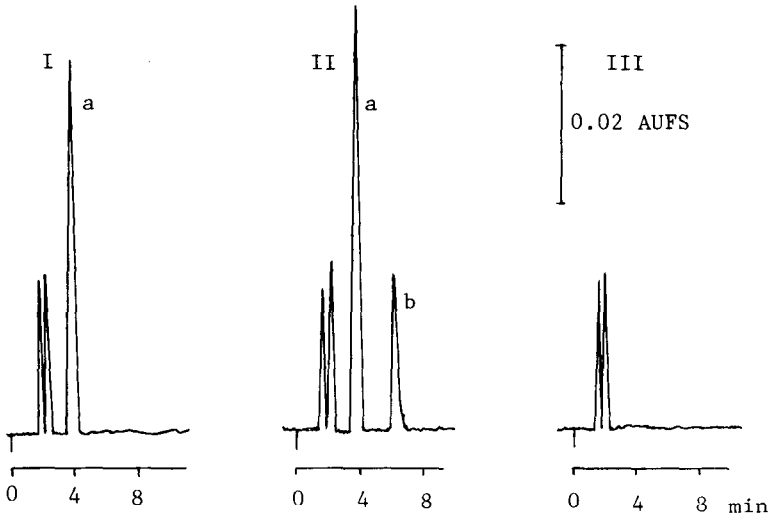

Fig. 2. Chromatograms of (I) an extract of liver homogenate sample obtained from a rat after the intraperitoneal administration of a single dose of acetaminophen (750 mg/kg); (II) an extract of the preceding liver sample (chromatogram I) containing the internal standard; (III) an extract of a liver sample from a control rat. Peaks: a = acetaminophen; b = sulfamerazine.

of them seemed to have been tested for such possibility of interference with acetaminophen and the internal standard used.

The successful use of the present assay was demonstrated by the results obtained in toxicokinetic studies conducted in two groups of male Sprague–Dawley rats treated with a single intraperitoneal dose of acetaminophen (750 mg/kg). Liver homogenates, 6 and 11 h following drug administration, were found to contain 129.54 g/g (S.E. = 30.92, n = 5) and 53.72 g/g (S.E. = 9.00, n = 5), respectively. A representative chromatogram which was thus obtained is shown in Fig. 2.

Pharmacokinetic analysis of acetaminophen serum concentrations as a function of time, following a single intraperitoneal dose of 750 mg/kg of body weight, has also been conducted in male Sprague- Dawley rats. The following results have been obtained from one animal (Fig. 3): k_{el} (the elimination rate constant) = 0.4 h^{-1}; k_a (the absorption rate constant) = 2.7 h^{-1}; V/F (the apparent volume of distribution over the fraction of the dose absorbed) = 1.2 l/kg; $AUC_{0-\infty}$ (the estimated total area under the drug serum concentration versus time) = 1622.9 $\mu g \cdot h/ml$; Cl/F (the total plasma clearance over the fraction of the dose absorbed, = dose/$AUC_{0-\infty}$) = 0.5 l/h · kg.

Applicability of our method to clinical use has also been evaluated with fresh human plasma. Spiked samples were used to construct a standard curve which showed similar linearity to the one made from rat serum data. Recovery is also very good, i.e. 100.3% (S.E. = 1.1, n = 5). Chromatographic elution is shown in Fig. 4. The sensitivity was improved by modifying the sample preparation and the chromatographic procedure. By pipeting 800 μl (instead of 600 μl) of the clear layer after acetaminophen extraction, and by reconstituting the residue after evaporation in 200 μl (instead of 300 μl), the

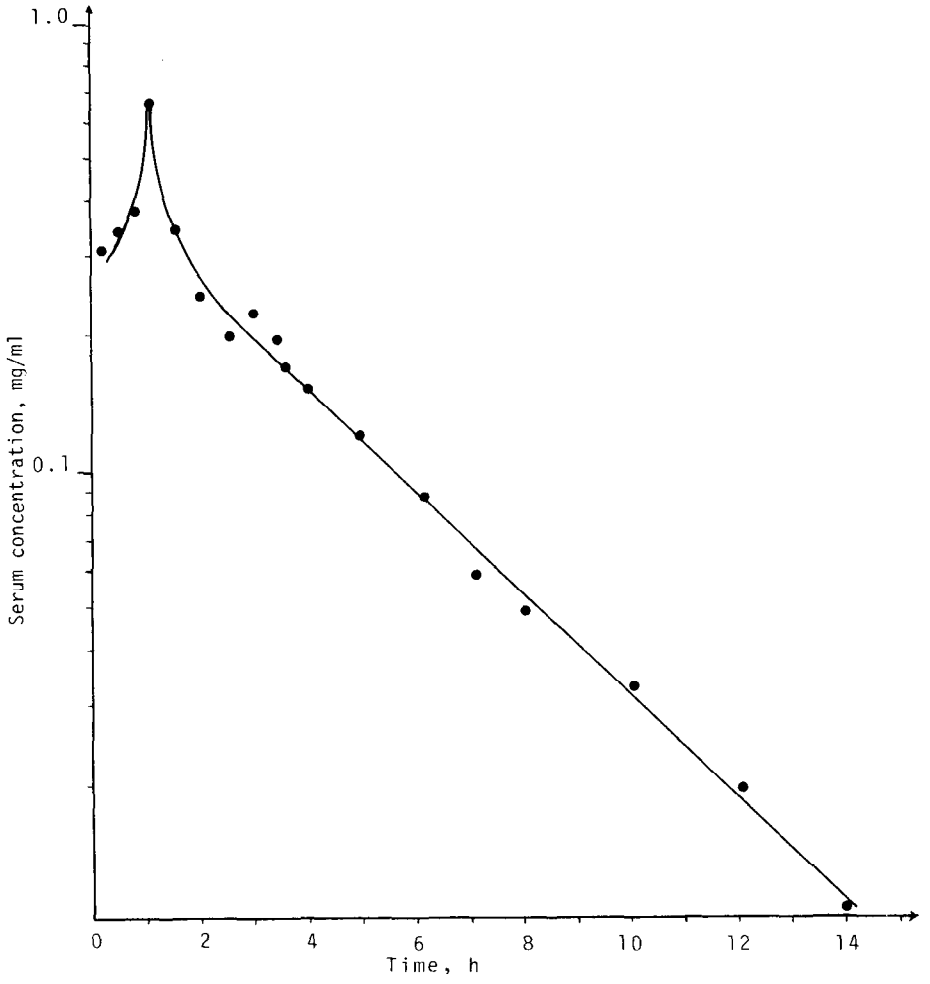

Fig. 3. Acetaminophen serum concentrations as a function of time after injection of a single intraperitoneal dose of 750 mg/kg to a male Sprague—Dawley rat.

sensitivity was found to be 0.025 μg/ml for a signal-to-noise ratio of 3. The recovery of the extraction, as estimated at 0.025 μg/ml of plasma, turned out to be 103.2% (S.E. = 2.5, n = 4). The standard curve was drawn by extraction of spiked standard plasma samples to cover the concentration range of interest (0.025, 0.050, 0.1, 0.5, 2.5, 5.0, 25.0, 50.0, 100.0 and 200.0 μg/ml). The average (± S.E.) equation of three calibration curves was found to be y = 0.0290 (± 0.0032)x + 0.2332 (± 0.0151) where y and x are defined as above. The within-day reproducibility was evaluated at 0.025 and 200 μg/ml of plasma and gave a coefficient of variation of 7.5 and 3.0%, respectively.

Advantages of the method

Many extraction techniques have been used to assay acetaminophen by HPLC [1—21]. Some of them require reciprocal shaking with fairly high volumes of organic solvents [2, 6, 10, 13, 16] which increase the cost of assay.

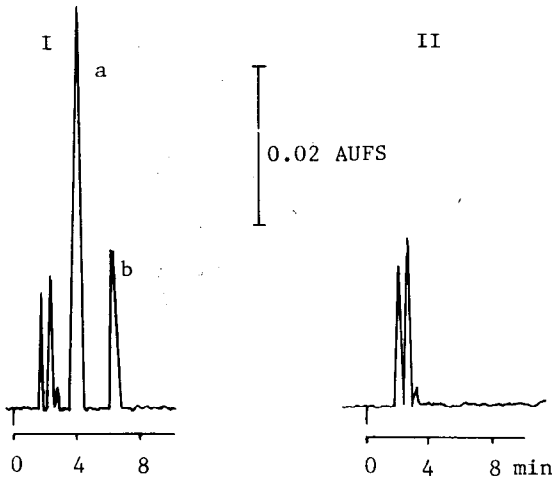

Fig. 4. Chromatograms of (I) extracted spiked human plasma sample containing acetaminophen at a concentration of 50 μg/ml and sulfamerazine at an equivalent concentration of 80 μg/ml; (II) extracted acetaminophen- and sulfamerazine-free human plasma sample. Peaks: a = acetaminophen; b = sulfamerazine.

Adjustment of plasma or serum pH, which was found to be unnecessary in our protocol, is frequently used to optimize non-ionisation and recovery of acetaminophen [8, 13, 16, 17]. Also, some investigators prefer to precipitate proteins with trichloroacetic acid, perchloric acid or a salt solution, and to inject the clear supernatant directly into the liquid chromatograph [5, 15, 18, 20]. These latter methods [5, 15, 18, 20] required a relatively large volume of plasma and/or gave a poor sensitivity. In our method, we use acetonitrile in the extraction solvent mixture (acetonitrile—isopropanol) to precipitate proteins and other macromolecules found in serum and tissue homogenates. The solvent mixture used was found to give complete recovery of acetaminophen when using a small volume of solvent of extraction and a short time of vortex mixing. With a mobile phase having a high water content (86%), the organic layer was no injected directly into the liquid chromatograph. In order to get the best peak shape of acetaminophen and sulfamerazine, a water solution was thus injected onto the column. Korduba and Petruzzi [10], who used a mobile phase different from ours, employed acetonitrile as the solvent of extraction and plasma protein precipitation. A few techniques carrying out extraction of acetaminophen from serum or plasma with an organic solvent do not use a recognized good solvent such as acetonitrile and methanol for protein precipitation [7, 9, 14, 16]. However, we found that the use of a good protein precipitation solvent [22] was generally decreasing the number and amplitude of contaminating peaks, especially in the case of tissue homogenates. The only reported liquid chromatographic method using UV detection for quantifying acetaminophen in tissue homogenates gives a very poor sensitivity [11].

Total analysis time for acetaminophen is 7.5 min, which is comparable to the most-rapid methods described in the literature [5, 12, 13, 21]. Tebbett et al. [21], with a less sensitive method, obtained a total elution time of 3 min but did not use an internal standard, which is preferable in order to decrease the

variations associated with extraction and injection. On the other hand, we have used an internal standard, sulfamerazine, which is readily available without prior synthesis.

The small volume of sample needed for analysis and the high sensitivity of the method (50 ng/ml) allow application in pharmacokinetic studies in small animals. The successful use of this technique in a toxicokinetic and organ distribution study in the Sprague—Dawley rat will be published elsewhere [23].

The preliminary results obtained with human plasma show that the method, besides being a rapid, sensitive and simple tool for kinetic studies, would be of considerable interest in clinical monitoring of acetaminophen, especially in the newborn where small volumes of blood have to be withdrawn.

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