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Determination of drugs by direct injection of plasma into a biocompatible extraction column based on a protein-entrapped hydrophobic phase

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ABSTRACT

Drugs were determined by direct injection of plasma samples into a biocompatible extraction column. The column is based on particles with a biocompatible external surface and a hydrophobic internal surface. The pores of the particles are small enough to exclude the protein molecules; the drug molecules can penetrate the porous particle and are retained on the hydrophobic internal surface. Biocompatibility of the particles was obtained by reaction of the external surface with the human plasma protein α_1-acid glycoprotein. The surface within the pores of the particles contains hydrophobic C_8 or C_{18} groups. The biocompatible extraction column was used in a fully automated system for the determination of ibuprofen, naproxen, propranolol, carbamazepine and phenytoin in plasma. No pressure increase was observed during the analysis of several hundred plasma samples. Plasma concentrations of propranolol in the range 4.5–125 ng/ml were determined with a precision (R.S.D.) of 0.75–1.8%. Linear calibration graphs were observed for the five drugs, and correlation coefficients of 1.0000 were obtained for four of the five model compounds.

INTRODUCTION

The determination of drugs, metabolites and endogenous compounds in biological fluids usually involves an isolation procedure prior to the chromatographic step. The most frequently used procedures are liquid–liquid extraction, protein precipitation and off-line solid-phase extraction with disposable extraction columns. All isolation methods are time consuming and introduce errors in the assay. The more manipulations the sample undergo prior to the quantification step, the lower are the accuracy and precision obtained. Therefore, it is advantageous to inject the sample directly into a liquid chromatographic system without off-line isolation procedures.

Different approaches have been used for direct injection of biological fluids [1,2]. The sample can be injected directly on to the analytical column [3–5], or by using a precolumn in the foreflush mode according to Arvidsson and co-workers [6,7] or in the backflush mode [8,9]. An early method describing the direct injection approach was published in 1978 [3]; Standard columns were utilized and very small sample volumes were injected, *i.e.*, 1–3 μl. A special type of column for the direct injection of biological fluids was developed by Hagestam and Pinkerton [4]; the surface chemistry within the pores is different to that on the external surface of silica particles.

One limitation with these types of columns is that the content of organic solvent that can be used in the mobile phase is limited. Concentrations higher than 15–20% precipitate plasma proteins, especially albumin, which also is present at a high concentration. Other similar phases have also been used [10]. By using the approach with a precolumn in the backflush mode, the compound of interest is extracted from the protein-containing biological sample and the

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precolumn is washed with a buffer. After washing, the solute is eluted from the extraction column on to the analytical column by pumping a mobile phase containing an organic solvent. As the plasma proteins do not come into contact with the organic modifier, there are no limitations concerning the concentration of organic modifier that can be used in this approach.

This paper describes a biocompatible trapping column that can be used as an extraction column for the direct injection of protein-containing biological fluids. The column is packed with silica particles having a biocompatible external surface and a hydrophobic surface within the pores. The external surface of the particles is covered with the human plasma protein α_1-acid glycoprotein (AGP), which makes the external surface of the particles compatible with the plasma proteins. The pores of the silica are small enough to exclude the plasma proteins.

EXPERIMENTAL

Chemicals and reagents

Methanol and acetonitrile of, HPLC grade were obtained from Lab-Scan (Dublin, Ireland). The drug compounds were gifts from drug companies. All other chemicals were of analytical-reagent grade.

Apparatus

The liquid chromatograph consisted of an LKB 2151 pump (Pharmacia–LKB Biotechnology, Uppsala, Sweden) and a Kontron Model 420 pump (Tegimenta, Rotkreutz, Swizerland), a Kontron Model 360 autosampler, equipped with 10- or 50-μl loops, or a manual Valco injector (Vici AG Valco, Schenkon, Swizerland), a C6W six-port switching valve with an electric acuator (Vici AG Valco), a Shimadzu (Kyoto, Japan) RF 535 fluorimeter and a Spectra 100 variable-wavelength UV detector (Spectra-Physics, San Jose, CA, USA). All experimental data were collected and analysed on a Kontron (Eching/Munich, Germany) 450 MT2 data system. This system also controlled the switching valve and the autosampler. The LKB 2151 pump (Pharmacia–LKB Biotechnology) was used for continuous pressure registration on both the analytical and the extraction column. The pressure was recorded with an LKB 2210 two channel recorder.

The following CT-sil columns (ChromTech, Norsborg, Sweden) were used in the study: CT-sil C_{18}, 100 × 4.6 mm I.D., 5 μm; CT-sil C_{18}, 150 × 4.6 mm I.D., 5 μm; and CT-sil C_8, 100 × 4.6 mm I.D., 5 μm. CT-sil C_8 or C_{18} guard columns (10 × 3.0 mm I.D., 5 μm) were used. The extraction columns were BioTrap Amine (for extraction of amines) and BioTrap Acid (for extraction of acids and non-protolytes), 10 × 3.0 mm I.D., from ChromTech. The filter holder in PEEK and the filter, used in front of the extraction column, were obtained from ChromTech.

Chromatographic system

On-line filter. Fig. 1A and B show schematic diagrams of the chromatographic systems used. The filter is used in the fully automated system (Fig. 1B) when the samples are injected with an autosampler. The filter is coupled between the injection valve and the extraction column in order to remove particles from the biological samples. The on-line filter is inserted in a special PEEK holder (ChromTech).

Extraction column. In the manual method the extraction column is a BioTrap Amine (for extraction of amines) or a BioTrap Acid (for extraction of acids or non-protolytes) column (10 × 3.0 mm I.D.). The column is coupled as a loop to a conventional loop injector as demonstrated in Fig. 1A. A volume of 1.5–2 ml of a buffer is injected on to the extraction column followed by an exact volume of a plasma sample. The buffer used to equilibrate the extraction column prior to injection differs for the different drugs. The methods for the different compounds are given in detail in the captions to the figures. After injection of the sample, the extraction column is washed with *ca.* 1.5 ml of the same buffer as was used for equilibration of the column prior to sample injection.

In the automated method, the extraction column can be coupled to a six-port switching valve with an electric actuator and an autosampler as demonstrated in Fig. 1B. The equilibration and the washing volumes are the same as for manual

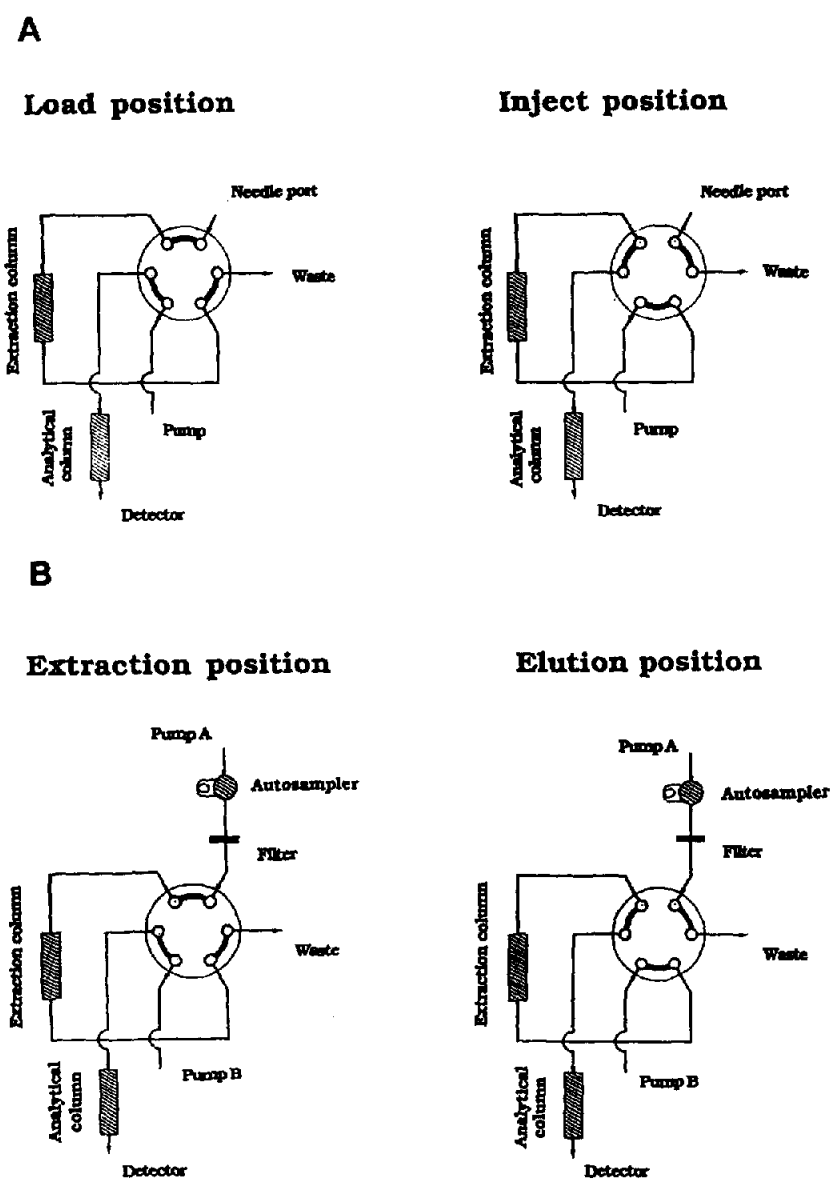

Fig. 1. (A) Schematic illustration of the chromatographic system used for manual injection with the BioTrap column coupled as a loop in a conventional injector. The plasma sample is injected in the load position. The plasma proteins are eluted by injection of buffer whereas the drug compound is enriched within the pores of the particles. After switching the valve to the inject position the sample is eluted (backflushing of the extraction column) on to the analytical column. (B) Schematic illustration of the fully automated system. The plasma sample is injected by an autosampler.

operation (see also the captions to the figures for the methods for the different drugs).

Guard column. The guard column is used to protect the analytical column from particles and endogenous compounds which are bound to the analytical column with high affinity. The guard column must be exchanged before it is overloaded with endogenous compounds otherwise these compounds are transported to the analytical column.

Calibration graphs, recovery and reproducibility studies

Calibration graphs were prepared by adding known amounts of the drug compounds to an exact volume of blank plasma. These samples were also used for the reproducibility studies. To evaluate the absolute recovery, known amounts of the different drug compounds were added to either blank plasma or to the mobile phase for the analytical column. The plasma samples were injected on to the extraction column and the peak areas were calculated. The samples dissolved in the mobile phase were injected directly on to the analytical column and the peak areas were determined. The absolute recovery was calculated by dividing the peak area obtained for the plasma samples with that obtained for the samples dissolved in the mobile phase and injected directly on to the analytical column. Plasma samples from patients and blank plasma were stored at −20°C.

Detailed descriptions of the conditions used for the determination of ibuprofen, naproxen, propranolol, phenytoin and carbamazepine in human plasma are given in the captions to the figures.

RESULTS AND DISCUSSION

The chromatographic systems described under Experimental and in Fig. 1A and B have been used for the determination of drugs in human plasma samples. The determination of drugs, metabolites and endogenous compounds in protein-containing biological fluids by direct injection of the sample into the liquid chromatographic system is very complicated owing to the high concentration of proteins and the large number of endogenous compounds present in this kind of sample. A hydrophobic silica such as a C_8 or a C_{18} matrix is not wetted at all when mixed with plasma, nor are the plasma proteins compatible with such a silica [6,11]. Particles with a biocompatible external surface that is wetted by the highly aqueous plasma sample have been obtained by covering the external surface of silica particles with AGP, which is a hydrophilic human plasma protein with a molecular mass of about 40 000. This protein was introduced in chromatography by Hermansson, who developed a chiral column based on the use of AGP as a chiral selector [12,13]. The protein is a glycoprotein containing five carbohydrate units constituting *ca.* 45% of the molecular mass. This protein is extremely stable in both native and immobilized forms. Immobilized AGP tolerates pure organic solvents such as alcohols and nitriles and can be used at extreme temperatures without being denatured [13]. The extraction column can be used with the mobile phases normally used for standard reversed-phase columns without affecting the performance of the extraction column.

Extraction column

Five different model compounds of different character were used, two carboxylic acids, the non-steroidal antiinflammatory drugs (NSAIDs) ibuprofen and naproxen, the β-blocker propranolol, an amine, and the weak acids phenytoin and carbamazepine, two antiepileptic drugs. The extraction column can be used in two different ways. The simplest way is to couple the column as a loop in a conventional loop injector according to Fig. 1A. It is also possible to fully automate the system using an autosampler and a switching valve as demonstrated in Fig. 1B. The sample is injected on to the extraction column, normally in volumes between 10 and 50 μl and sometimes larger, *e.g.*, 100 μl. Carboxylic acids and also weaker acids are extracted at a pH 2.1–6. Basic drugs are extracted at pH ≤7.5, normally between pH 6 and 7.5. Higher pH cannot be used because the column is silica based. The pH to be used during extraction of a drug compound depends on the pK_a value of the drug and how the recoveries of the endogenous compounds are affected by the pH.

After injection of the serum or plasma sample on to the extraction column, the plasma proteins are washed out by pumping 1.1–2 ml of extraction mobile phase. After washing, the sample is eluted from the extraction column with the mobile phase for the analytical column in the backflush mode, by switching the manual injector to the inject position or by switching the six-port valve (see Fig. 1A and B). The complete methods for the different drug compounds are

given in detail under Experimental and in the captions to the figures.

Two different extraction columns were used, BioTrap Acid C_{18} (for extraction of acidic and non-protolytic drugs) and BioTrap Amine C_{18} (for extraction of basic compounds). The external surfaces of the two extraction columns are identical, but the internal surface of the BioTrap Amine column is end-capped in order to minimize the band broadening in the extraction column. The extraction column has the dimensions 10×3.0 mm I.D., which means that the amount of packing material in the column is small. The reasons for using a short extraction column are to shorten the washing time and to shorten the time for re-equilibration with the extraction buffer, after the backflushing of the extraction column with the mobile phase containing an organic modifier. This means that the total time of analysis, including extraction and analysis, can be short.

Plasma and serum contain a very large number of endogenous compounds of different kinds, both low-molecular-mass compounds of different hydrophobicity and macromolecules. The endogenous compounds, drugs and metabolites are retained on the extraction column and the amount extracted is affected by the affinity to the plasma proteins and the affinity to the internal surface of the extraction column. Backflushing the extraction column with a mobile phase containing an organic modifier elutes the compound of interest together with endogenous compounds. Some endogenous compounds in plasma are highly retained on the extraction column even when the column is eluted with a mobile phase containing a high concentration of organic modifier. This means that after injection of a certain amount of samples the extraction column must be replaced. The extraction column will be deactivated by adsorption of endogenous compounds with high affinity and they are not completely washed out on backflushing the column. This gives a broadening of the peaks on the analytical column because the sample is spread out in the extraction column, giving a broad starting zone on the analytical column. The number of samples that can be injected before replacement of the extraction cartridge is necessary depends on the mobile phases used in the extraction and analytical columns, and also the volume of serum or plasma that has been injected. By injection of <20 μl of plasma samples it is possible to inject several hundred samples before replacement of the small extraction cartridge. This means a low cost per sample compared with other isolation procedures such as the use of disposable off-line extraction cartridges and liquid–liquid extraction, both of which are very time consuming procedures.

The described method can also be fully automated. One of the major problems with direct injection of protein-rich samples is that the proteins precipitate in the column, either in the filter or when they come into contact with the hydrophobic packing material [6,11]. It is well known that sintered stainless-steel frits precipitate plasma proteins [6]. This results in increased pressure on repeated injections of this kind of sample.

Another problem is the decrease in the separation efficiency of the analytical column because endogenous compounds are transferred to and adsorbed on the analytical column. This can be avoided by changing the guard column coupled in front of the analytical column before it is overloaded with endogenous compounds.

Table I demonstrates how the pressure over the extraction column and the analytical column is affected by the injection of 465 plasma samples (10 μl) containing ibuprofen. The effects on the number of theoretical plates, the retention time and the peak symmetry are also included in

TABLE I

STABILITY OF THE SYSTEM

Analyte: ibuprofen (4.99 μg/ml) injection volume: 10 μl.

Parameter	Sample No.	
	1	465
Pressure, analytical column (bar)	74	73
Pressure, extraction column (bar)	0	0
t_r (min)	3.63	3.50
N	4004	3804
Asymmetry factor	1.27	1.12

Table I. As can be seen, no pressure increase was obtained on the extraction column and the analytical column after injection of 465 plasma samples, nor were the other parameters significantly affected. It can also be mentioned that no guard column was used in front of the analytical column during this experiment.

It was observed that the ibuprofen peak shape was affected to some extent after 200–300 plasma samples. Therefore, the analytical column was backflushed once for about 2–5 min during the analysis of the 465 plasma samples. However, during routine use it is recommended to use a guard column which is exchanged before it is overloaded with endogenous compounds.

During this study the samples were analysed with the fully automated system as shown in Fig. 1B. After injection of the 465 plasma samples, six standard samples, in the therapeutic concentration range, between 0.52 and 40.4 μg/ml, were injected to test whether the calibration graph was linear. The linear regression equation was $y = 0.2016 + 3.6411\ x$ (y = peak area; x = concentration in μg/ml) and the correlation coefficient was 1.000, which clearly demonstrates that the extraction column had not deteriorated and gave a constant recovery over the whole concentration range.

Recovery

The retention of a solute on a reversed-phase extraction column can be expressed by

$$k'_{(s)} = qD_{(s)} \quad (1)$$

where q is the phase ratio and $D_{(s)}$ is the distribution ratio of the solute between the solid and the mobile phase. However, injection of plasma samples containing large amounts of proteins, such as albumin and AGP, involved in the binding of drugs and endogenous compounds affects the chromatographic properties of the low-molecular-mass solutes. Albumin is the drug-binding protein that is present in plasma at the highest concentration and it is involved in the binding of many different types of drugs. Albumin has a molecular mass of about 66 000 with the dimensions 150 × 38 Å [14]. The Stokes radius has been determined to be 33.4 Å [14], which means that a pore with a diameter of about 60 Å will exclude the albumin molecules from the internal surface of the particles. AGP has a larger Stokes radius than albumin. If a certain solute (S) is bound to only one binding site on albumin (P), and if the protein is excluded from the pores and the binding of the protein to the external surface of the particles in the extraction column can be neglected, the distribution ratio, D_s, of the solute (S) can be expressed by

$$D_S = [SA_S]/([S] + [PS]) \quad (2)$$

where [S] and [PS] are the concentrations of the free drug and the drug–protein complex, respectively, and $[SA_S]$ is the concentration of the drug bound to the binding site, A_S, on the internal surface of the particles in the extraction column.

An expression for the retention of a solute in a sample containing proteins can be derived from eqns. 1 and 2:

$$k'_{(S,P)} = k'_{(S)}/(1 + [PS]/[S]) \quad (3)$$

where [PS]/[S] is the drug–protein binding ratio and $k'_{(S)}$ is the retention of the solute (S) in the absence of proteins. From eqn. 3, it can be seen that the retention of the solute in the sample zone decreases on increasing the drug–protein binding ratio, [PS]/[S]. This means that the sample migrates a longer distance in the extraction column if the sample contains proteins. If a short extraction column is used, a high recovery will be obtained if the drug–protein binding ratio is not too high and/or if an extraction column with high hydrophobicity is used.

In the chiral analysis of enantiomers in plasma, it is very important to have a high recovery in the isolation step independently of whether liquid–liquid extraction, precipitation of plasma proteins or an off-line or on-line extraction column are used. As enantiomers are bound stereoselectively to the plasma proteins, *i.e.*, the [PS]/[S] ratio differs for enantiomers, the retention, $k'_{(S,P)}$, according to eqn. 3 for the enantiomers is different. From this it follows that the concentration ratio between enantiomers, determined by the chromatographic method, can

be affected by the isolation step if an isolation procedure giving low recovery is used. The enantiomer with the highest affinity to the plasma proteins gives the lowest recovery.

The absolute recoveries of the drug compounds were determined by comparing the peak areas of three spiked plasma samples and three reference samples at different concentrations. The reference samples were injected directly on to the analytical column and the spiked plasma samples were injected on to the extraction column coupled to the analytical column through the switching valve. The results are summarized in Table II. The absolute recoveries vary between 78 and 96% for the five different drug compounds. The lowest recovery was obtained for propranolol because it is extracted at pH 7, which means that it is fully ionized. Using a higher pH for extraction increases the recovery. However, a silica-based column cannot be used at such a high pH that reduces the charge on the propranolol molecules ($pK_a = 9.5$).

The reproducibility of the recovery for three different extraction columns, produced from three different batches of packing material, was also tested using ibuprofen as a model compound, and a mean recovery of 96.2% was obtained with a relative standard deviation (R.S.D.) of 2.0%. When determining the absolute recovery it is very important to be sure that the reference sample is not adsorbed on the walls of the glassware, plastic tubes or containers used for the reference samples. The adsorption of a compound on the walls of a vial or other container depends on the nature of the material in the container, the nature of the compound and the solvent used to dissolve the compound. For example, when the propranolol reference sample was dissolved in phosphate buffer (pH 7.4), recoveries between 110 and 130% were obtained. This was the result of adsorption of the relatively hydrophobic propranolol on the polypropylene vials. Using such a reference sample and such vials gives a too small peak area of the reference sample and a too high a recovery. By dissolving propranolol in the mobile phase containing 33% acetonitrile this problem with adsorption on the vials was avoided. No indications of adsorption of propranolol on the polypropylene vials from the plasma samples were observed. This is most likely the result of the protein binding and the presence of endogenous compounds in the plasma sample which compete with the drug for binding to the walls of the vials.

Precision

The intra- and inter-day variations were determined for the various compounds and the results of the former studies are summarized in Table III. The studies were performed in a concentration range normally obtained after administration of a single dose of the different drugs, *i.e.*, the therapeutic concentrations are higher. It can be seen that a very good precision can be obtained even at the lowest concentration levels studied. Table IV summarizes the inter-day variations. This study was performed in the same concentration interval as in the intra-day study. The inter-day variation was determined at

TABLE II

ABSOLUTE RECOVERIES FROM PLASMA

Compound	Injection volume (μl)	Concentration (μg/ml)	Recovery (%)	R.S.D. (%)[a]
Carbamazepine[b]	50	8.1	89.7	1.2
Phenytoin[b]	50	13.3	84.1	2.1
Propranolol[c]	50	$11.6 \cdot 10^{-3}$	77.8	4.5
Ibuprofen[b]	10	12.5	96.0	1.5

[a] $n = 3$.

[b] BioTrap Acid extraction column (see Experimental).

[c] BioTrap Amine extraction column (see Experimental).

TABLE III

INTRA-DAY VARIATIONS

Compound	Concentration (μg/ml)	R.S.D. (%)[a]
Ibuprofen	0.52	6.7
	3.11	2.0
	40.4	0.96
Naproxen	1.05	2.80
	5.27	0.44
	10.5	1.18
	21.1	0.52
	42.1	0.62
Propranolol	$4.45 \cdot 10^{-3}$	1.8
	$8.92 \cdot 10^{-3}$	1.0
	$22.3 \cdot 10^{-3}$	0.75
	$53.5 \cdot 10^{-3}$	1.54
	$89.2 \cdot 10^{-3}$	1.40
	$124.8 \cdot 10^{-3}$	1.54
Phenytoin	1.76	2.92
	13.5	1.03
	30.0	0.92
Carbamazepine	2.05	0.73
	5.13	1.00
	10.3	0.31
	15.2	0.54
	20.5	0.49

[a] $n = 6$.

two different concentrations of each drug. As can be seen from Table IV, a high inter-day reproducibility was obtained for the different drugs.

TABLE IV

INTER-DAY VARIATIONS

Compound	Concentration (μg/ml)	R.S.D. (%)	n
Ibuprofen	3.24	3.00	6
	14.9	1.92	6
Propranolol	$4.11 \cdot 10^{-3}$	4.58	6
	$36.6 \cdot 10^{-3}$	2.36	6
Phenytoin	7.63	2.49	5
	23.8	1.21	5
Carbamazepine	5.27	1.28	5
	15.8	1.42	5

Determinations

Manual isolation procedures such as liquid–liquid extraction, the use of off-line extraction cartridges or precipitation of plasma proteins followed by centrifugation are very time consuming. This type of isolation procedure also introduces errors in the determination that decrease both the accuracy and precision. Therefore, a method that requires a minimum of manipulations of the sample prior to the quantification step is to be preferred. This is of special importance, of course, in large clinical projects where several thousand plasma samples must be analysed with high precision and accuracy.

Ibuprofen. Ibuprofen was determined using the method described under Experimental and in Fig. 1. A representative chromatogram of a plasma sample from a patient who had received a single oral dose of 200 mg of ibuprofen is demonstrated in Fig. 2. No disturbing endogenous peaks were detected, owing to the high detection selectivity obtained with fluorimetric detection. The maximum sensitivity was obtained using an excitation wavelength of 227 nm and an emission wavelength of 555 nm. However, at high drug concentrations these wavelengths gave too high a response and therefore wavelengths of 225 and 535 nm, respectively, were used. Plasma concentration *versus* time curves for two patients are presented in Fig. 3. Ibuprofen was also determined using the manual method, by coupling the extraction column to a conventional loop injector according to Fig. 1A, which works well when the number of samples is so small that automation is unnecessary.

Naproxen. Naproxen is a well known NSAID and can easily be determined using the described method with either UV or fluorimetric detection. Fig. 4 shows a chromatogram of naproxen using UV detection at 328 nm. No disturbing endogenous compounds are detected, because a high detection selectivity is obtained using this wavelength. Using fluorimetric detection with an exitation wavelength of the 230 nm and an emission wavelength of 350 nm a very high detection selectivity and a very high sensitivity can be obtained. Using UV detection at 328 nm it is necessary to inject about 50 μl of sample. By using fluorimetric detection and if the fluorime-

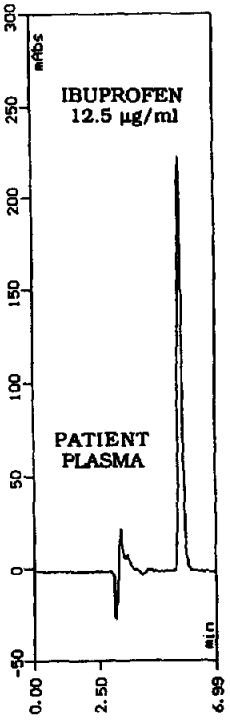

Fig. 2. Isolation of ibuprofen from human plasma after administration of a single oral dose of 200 mg. Chromatographic conditions: injection volume, 10 μl; extraction column, BioTrap Acid C_{18} (10 × 3.0 mm I.D.); extraction buffer, 0.20 *M* phosphate buffer (pH 2.1); flow-rate, 0.55 ml/min; extraction position, 2 min; analytical column, CT-sil C_{18} (100 × 4.6 mm I.D., 5 μm) equipped with a CT-sil C_{18} guard column (10 × 3.0 mm I.D., 5 μm); mobile phase, methanol–82 m*M* phosphate buffer (pH 6.0) (65:35); flow-rate, 1.0 ml/min; elution position, 2 min; fluorimetric detection (exitation wavelength 225 nm, emission wavelength 535 nm).

ter is operated in the low-sensitivity mode, 1 μl can be injected. Such small volumes are difficult to handle with conventional autosamplers. In order to obtain an optimum method for naproxen using fluorimetric detection, the excitation and/or the emission wavelengths must be adjusted to decrease the sensitivity so that 10-μl plasma samples can be injected.

Propranolol. Propranolol was extracted from plasma samples using a BioTrap Amine C_{18} column and a pH of the extraction mobile phase of 7. This is not the optimum pH for extraction as propranolol has a pK_a value of >9, which means that it is fully protonated at pH 7.

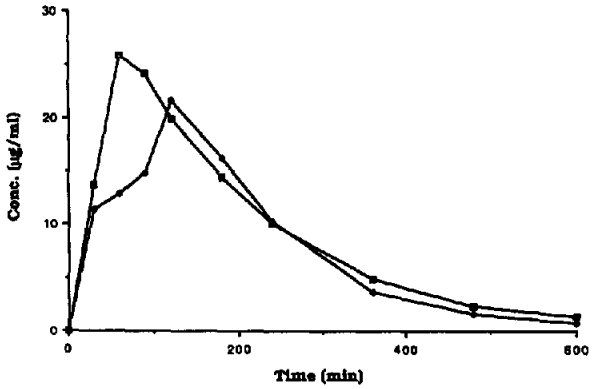

Fig. 3. Plasma concentration *versus* time curves for ibuprofen in samples from two patients after administration of a 200-mg single oral dose.

However, a recovery of about 80% was obtained despite the low extraction pH. Propranolol was detected using fluorescence detection with an excitation wavelength of 200 nm and an emission wavelength of 340 nm. This gives a high detection selectivity and a high sensitivity, making possible the determination of propranolol in

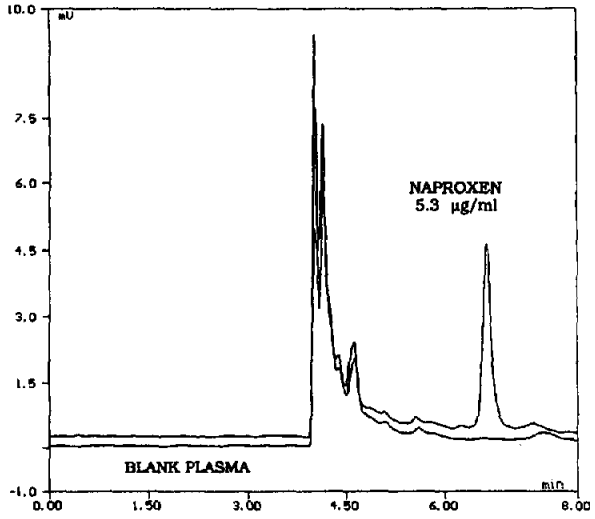

Fig. 4. Isolation of naproxen from spiked human plasma. Chromatographic conditions: injection volume, 50 μl; extraction column, BioTrap Acid C_{18} (10 × 3.0 mm I.D.); extraction buffer, 0.20 *M* phosphate buffer (pH 2.1); flow-rate, 0.55 ml/min; extraction position 3 min; analytical column, CT-sil C_{18} (100 × 4.6 mm I.D., 5 μm); mobile phase, methanol–0.12 *M* phosphate buffer (pH 3.0) (65:35); flow-rate, 1.0 ml/min; elution position, 4 min; UV detection at 328 nm.

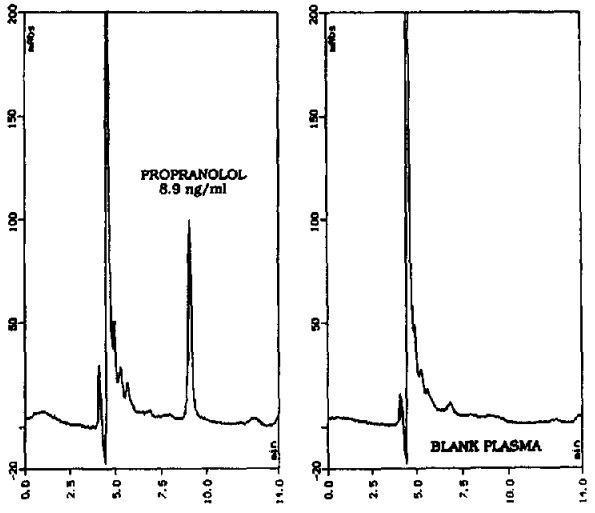

Fig. 5. Isolation of propranolol from spiked human plasma. Chromatographic conditions: injection volume, 50 μl; extraction column, BioTrap Amine C_{18} (10 × 3.0 mm I.D.); extraction buffer, 48 m*M* phosphate buffer (pH 7.0); flow-rate, 0.55 ml/min; extraction position, 3 min; analytical column, CT-sil C_8 (100 × 4.6 mm I.D., 5 μm); mobile phase, acetonitrile–0.12 *M* phosphate buffer (pH 3.0) (28:72); flow-rate, 1.0 ml/min; elution position, 6 min; fluorimetric detection (excitation wavelength 220 nm, emission wavelength 340 nm).

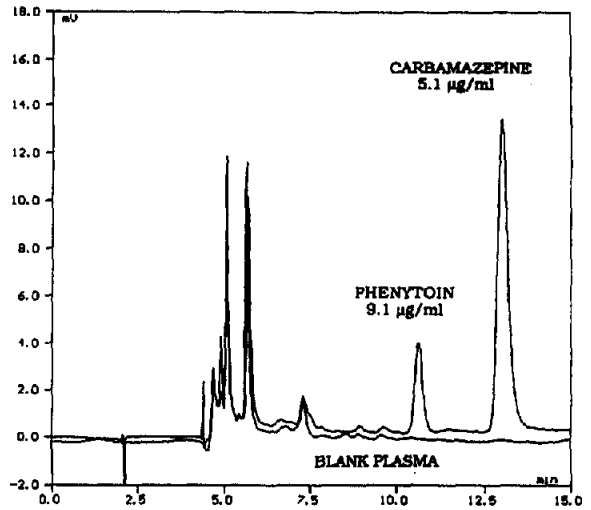

Fig. 6. Isolation of phenytoin and carbamazepine from spiked human plasma. Chromatographic conditions: injection volume, 50 μl; extraction column, BioTrap Acid C_{18} (10 × 3.0 mm I.D.); extraction buffer, 82 m*M* phosphate buffer (pH 6.0); flow-rate, 0.55 ml/min; extraction position, 3 min; analytical column, CT-sil C_{18})150 × 4.6 mm I.D., 5 μm) equipped with a CT-sil C_{18} guard column (10 × 3.0 mm I.D., 5 μm); mobile phase, methanol–82 m*M* phosphate buffer (pH 6.0) (50:50); flow-rate, 1.0 ml/min; elution position, 6 min; UV detection at 254 nm.

plasma samples after administration of a single oral dose of about 40 mg of propranolol hydrochloride. Fig. 5 shows representative chromatograms of blank plasma and propranolol in a spiked human plasma sample using the described method.

Phenytoin and carbamazepine. The antiepileptic drugs phenytoin and carbamazepine, at therapeutic concentrations, can be determined simultaneously by the described method using UV detection at 254 nm, a BioTrap Acid C_{18} column for extraction and injection of 50 μl of plasma. A chromatogram is presented in Fig. 6 together with that for a blank plasma sample. The sensitivity obtained at 254 nm is good enough to detect carbamazepine in the therapeutic concentration range. Detection of carbamazepine at 285 nm gives about a 90–100% higher sensitivity than at 254 nm, which means that it is sufficient to inject only 10 μl of plasma. By using this method the carbamazepine concentrations in several hundred of plasma samples have been determined without disturbances. This method is also rapid, with a retention time of 5.7 min for carbamazepine. It has been observed that by using an extraction mobile phase with a pH of 2.1 the system was more stable compared with a pH of 6. However, more endogenous compounds were detected using pH 2.1.

Calibration graphs

Calibration graphs in the therapeutic concentration range were obtained for all the drug compounds studied. The slopes and the intercepts, with the confidence limits at $P = 0.05$, of the linear regression equations are presented in Table V together with the correlation coefficients. Correlation coefficients of 1.0000 were obtained for four of the five model compounds. The calibration graphs cover both sub-therapeutic and therapeutic concentration ranges.

CONCLUSIONS

A biocompatible extraction column has been developed. The internal surface of the particles

TABLE V

CALIBRATION GRAPHS: LINEAR REGRESSION ANALYSIS

Compound	Concentration interval (μg/ml)	n^a	Slope and confidence limits ($P = 0.05$)	Intercept	Correlation coefficient
Ibuprofen	0.52–40.4	6	3.6411 ± 0.0458	0.2016 ± 1.2305	1.0000
Naproxen[b]	1.05–42.1	5	0.1216 ± 0.0010	0.0026 ± 1.5342	1.0000
Phenytoin	4.56–30.0	5	0.0907 ± 0.0019	0.0047 ± 1.8504	0.9999
Carbamazepine[c]	2.05–20.5	5	0.6671 ± 0.0079	−0.0204 ± 1.7896	1.0000
Propranolol	$(4.5–125) \times 10^{-3}$	6	2.4981 ± 0.0165	−0.0885 ± 1.3555	1.0000

[a] Number of calibration points.
[b] UV detection at 328 nm, 50 μl injected.
[c] UV detection at 254 nm, 50 μl injected.

is hydrophobic as it is covered by alkyl chains of different length, whereas the external surface is covered with the human plasma protein AGP in order to make the surface compatible with the plasma proteins. Acidic and non-protolytic samples are extracted using a BioTrap Acid C_{18} column, whereas basic drugs are extracted using a BioTrap Amine C_{18} column. Several hundred plasma samples can be injected without a pressure increase or affecting the chromatographic properties of the column. The intra- and interday precisions are high and calibration graphs with excellent linearity were obtained. A correlation coefficient of 1.0000 was obtained for four of the five model compounds.

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