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On-line radiometric determination of [^{14}C]-urapidil and its main metabolites in rat plasma, using post-column ion-pair extraction and solvent segmentation techniques*

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Abstract: Post-column ion-pair extraction has been applied to the on-line radiometric determination of [^{14}C]-urapidil and its main metabolites in reversed-phase liquid chromatography (LC). [^{14}C]-remoxipride was used as internal standard. Chromatography was performed isocratically, using acetonitrile–water (pH = 2.2) as eluent on a cyanobonded column. Various ion-pair reagents were tested for their extraction efficiency of the analytes mentioned from the eluent into chloroform. Quantitative extraction was possible using sodium dodecylbenzenesulphonate as ion-pair reagent. The influence of the extraction solvent composition, the percentage organic modifier in the eluent and the flow-rates of eluent and scintillator on the extraction percentage and the ^{14}C-counting efficiency has been studied. In addition, the enhancement of sensitivity is possible using an on-line radiometric procedure. Finally, the method has been applied to the analysis of rat plasma for [^{14}C]-labelled urapidil and its metabolites.

Keywords: [^{14}C]-*urapidil; reversed-phase LC; post-column ion-pair extraction; on-line radiometric detection.*

Introduction

In pharmaceutical and biochemical studies it can be advantageous to use radiolabelled compounds because radioactivity determination is relatively simple to perform, and provides accurate information on the distribution, excretion and recovery of the compounds of interest. Quantification of the metabolites is straightforward because the radioactivity signal only depends on the determination of the radionuclide applied (e.g. ^{3}H, ^{14}C, ^{35}S, ^{32}P, ^{125}I).

For the radioactivity determination in LC eluents of the nuclides mentioned above, three detection methods are in common use [1]. The LC eluent can be fractionated and, after addition of liquid scintillator, counted in a scintillator counter (off-line detection).

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Alternatively, the liquid scintillator can be pumped continuously into the LC eluent and the mixture led through the flow-cell of a radioactivity monitor (homogeneous on-line detection). Finally, the eluent can be pumped through the radioactivity monitor, the flow-cell of which is packed with solid scintillator material (e.g. europium-activated granular CaF_2, or yttrium silicate; heterogeneous on-line detection).

It is evident that the radioactivity detection limit strongly depends upon the counting time T_d [2]. This probably explains the widespread use of off-line detection.

In on-line detection, the counting time equals the mean residence time in the flow-cell of the detector, which is given by:

$$T_d = V_d/F_t \tag{1}$$

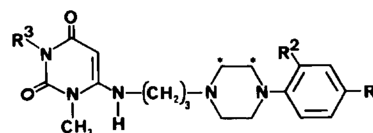
in which V_d = the effective flow-cell volume (in ml) and F_t = the total flow-rate through the flow-cell (in ml min^{-1}). V_d is optimized so as to preserve the chromatographic integrity in the radiogram.

Lowering the eluent flow-rate is not a viable alternative as the analysis time will increase proportionally and, because of band broadening in the capillaries and detector at low flow-rates [3], the possible gain in counting time will be limited. It therefore seems desirable to first scan the radioactivity distribution in the radiogram during the separation procedure (direct measurement) and next, if necessary, to count one or more regions in the radiogram at enhanced counting time in a partially off-line detection mode.

For this purpose, a method was introduced for on-line radioactivity monitoring of LC eluents in reversed-phase LC, based on the use of a water-immiscible liquid scintillator [4]. The scintillator is continuously pumped to the LC eluent and analytes are extracted into the scintillator phase. A solvent-segmented flow pattern is obtained which is led through the radioactivity monitor (direct measurement) and stored in a capillary storage loop. After storage of the complete chromatogram, the contents of the loop are introduced into the monitor again at a flow-rate that can be varied according to the counting times desired, as decided from the radioactivity distribution recorded in the direct measurement (reverse measurement). Because of the small range of the beta-particles emitted by the radioactive decay compared to the length of the aqueous and scintillator segments formed, the counting efficiency E strongly depends on the extraction percentage of the analytes of interest from the eluent into the water-immiscible liquid scintillator. Extraction percentages are poor when analytes are ionized under the chromatographic separation conditions, so that the extraction and segmentation principle cannot be used directly.

The possibility of increasing the extraction percentage of remoxipride (a potential basic pharmaceutical) by a post-column pH increase of the eluent, before adding the scintillator, was explored recently [5]. In the present work ion-pair extraction techniques for the determination of [^{14}C]-urapidil and its main metabolites in rat plasma have been investigated. Urapidil (Ebrantil) is used in the oral treatment of hypertension and the treatment of hypertensive crises by intravenous bolus injection [6, 7]. Urapidil and its metabolites are protonated under acid conditions (pH <3). For structural formulae, see Fig. 1.

This paper deals with the optimization of the ion-pair extraction of urapidil, its metabolites and the internal standard, the influence of the scintillator/eluent flow-rate ratio on the post-column extraction and sensitivity of on-line radioactivity detection, and



	R^3	R^2	R^1
urapidil	CH_3	OCH_3	H
M1	CH_3	OCH_3	OH
M2	CH_3	OH	H
M3	H	OCH_3	H

remoxipride :

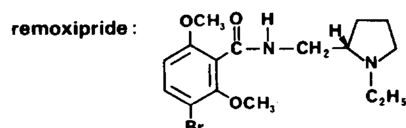


Figure 1
Structural formulae for urapidil, its metabolites M1, M2, M3 and the internal standard remoxipride.

the use of the reverse measurements. Finally, the principle is applied to the determination of urapidil and its metabolites in rat plasma samples.

Experimental

Apparatus

The schematic diagram of the system is given in Fig. 2. The liquid chromatograph was a model series 3B dual-head reciprocing pump (Perkin–Elmer, Norwalk, CT, USA), equipped with a model 7126 injector (Rheodyne, Cotati, CA, USA), an Uvikon model 725 UV detector (Kontron, Zürich, Switzerland), a model 12611 (8-port) switching valve (Valco, Houston, TX, USA) and a laboratory designed radioactivity monitor, respectively. The injector and switching valve were used with model 7163 solenoid valve kits (Rheodyne). All capillaries were made of stainless steel (type 304 or 316). The LC eluent was mixed with the water-immiscible scintillator in a model ZT 1 C mixing T-

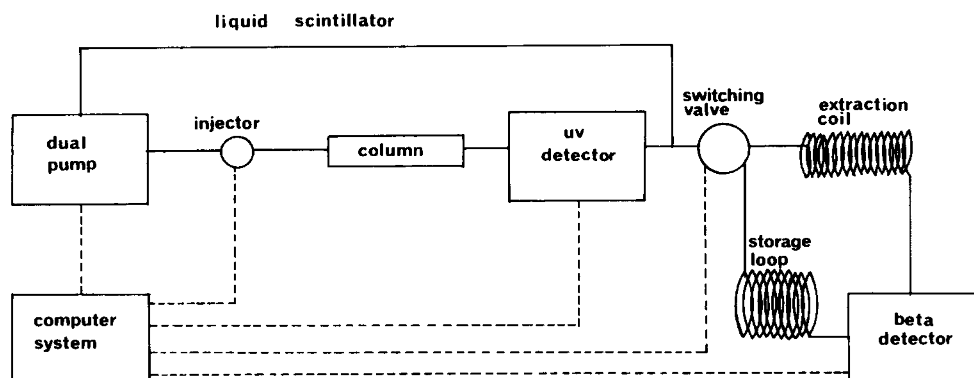


Figure 2
Schematic diagram of LC equipment with extraction/storage system and on-line radioactivity determination.

piece (1/16″ × 0.25 mm, Valco). In this work, two extraction coils were used: 0.42 or 1.1 ml volume with 0.75 mm i.d. and a coil diameter of 1.0 and 1.5 cm, respectively. The dimensions of the coiled storage loop were: 50 m × 1/16″ o.d. × 1.0 mm i.d., coil diameter 25 cm. The outlet capillary from the liquid scintillator pump was 0.25 mm i.d. (length 80 cm). For F(scintillator) >50 μl min^{-1}, this provided sufficient back pressure, which was found essential for the pumping accuracy.

The radiomonitor consisted of a laboratory-built light-tight housing, an isoflo flow-cell (Nuclear Enterprises, Edinburgh, UK) which is a cylindric quartz tube with a geometric volume of 63 μl, two model 9849A photomultiplier tubes (EMI THORN, Middlesex, UK), a model 456 high voltage power supply, two model 265 photomultiplier bases, two model 218 magnetic shieldings, two model 113 scintillator preamplifiers (Ortec, Oak Ridge, TN, USA), two model 2110 timing filter amplifiers and two model 1428A constant-fraction discriminators (Canberra, Meriden, CT, USA). The outputs of the discriminators can be sent to both laboratory-devised coincidence counter/timer interface in the computer system and, simultaneously, to a model 418A coincidence unit, equipped with a model 431 timer/scaler (Ortec). For controlling the chromatographic apparatus, data acquisition and data processing, a computer system was used with laboratory devised soft-ware. It consisted of a DATA-RAM LSI-11/2 computer with a DEC LSI-11/2 u-processor under RT-11 (Digital Equipment Corp., Maynard, MA, USA). Furthermore, a model 862 interface (Nelson Analytical, Cupertino, CA, USA), a model 910 terminal (Televideo, Sunnyvale, CA, USA) and a model MX-100 III printer (Epson, Nagano, Japan) were used.

Columns and mobile phases

Separations were performed using Brownlee cyano Spheri-5 cartridge columns (100 × 4.6 mm) (Brownlee Labs, Santa Clara, CA, USA) or Pierce cyano Spheri-5 cartridge columns (100 × 4.6 mm) (Pierce Chemical Comp., Rockford, IL, USA). For injection of plasma samples, in some cases a cyano-bonded guard column was used (15 × 3.2 mm, connected directly to the analytical column). For mobile phases, HPLC-grade water and acetonitrile (Fisons, Scientific Equipment Div., Loughborough, UK), orthophosphoric acid (Baker Chemicals, Deventer, The Netherlands) and sodiumhexylsulphonate (Alltech Europe, Eke, Belgium) were used.

Chemicals and standards

Ready Solv NA (Beckman Instruments, Fullerton, CA, USA) was used as a water-immiscible liquid scintillator. For absolute activity determination of standards and plasma samples. Instagel (United Technologies Packard, Warrenville IL, USA) was used. Absolute activities were determined by using the linear extrapolation method as described in [8]. Urapidil, [^{14}C]-urapidil [specific activity: 1.01 GBq/mmol, radiochemical purity 87.9% (by radio-TLC)], the metabolites M1, M2 and M3 and rat plasma samples were received as a gift from Byk Gulden Pharmazeutika (Konstanz, FRG). Plasma samples were taken at different time intervals after oral administration of [^{14}C]-urapidil (specific activity: 0.48 MBq $mmol^{-1}$, dose 5 mg kg^{-1}) to male SD rats, and stored at −18°C until analysed. Remoxipride and [^{14}C]-remoxipride (specific activity: 1.06 GBq $mmol^{-1}$, radiochemical purity: 96.7%) were gifts from Astra Alab AB (Sodertalje, Sweden). The ^{14}C-labelled standards were diluted in acetonitrile and stored at −18°C. All other standards and stock solutions were stored at 4°C. The following materials were tested as ion-pairing agents: sodium dodecylbenzenesulphonate (DBS,

80–85%, Koch-Light Lab., Colnbrook, UK) sodium dodecylsulphonate (DDS, practical, Eastman Kodak, Rochester, NY, USA); sodium hexylsulphate (practical, Eastman Kodak); sodium naphthalene-2-sulphonate (technical, Fluka, Buchs, Switzerland) and sodium 9,10-dimethoxyanthracene-2-sulphonate (DAS, purum, Fluka). Furthermore, chloroform, *o*-xylene (Baker) and Ready Solv NA were applied as extraction solvents. All other chemicals used were of analytical grade.

Batch extraction procedure

For the determination of the extraction recovery of urapidil, its metabolites and remoxipride as a function of eluent and extraction solvent composition, the following procedure was normally used. Equal volumes of eluent (spiked with known amounts of the analytes) and extraction solvent were shaken for 1 min in a separatory funnel. Phase separation was accelerated by centrifugation of the total volume in a centrifugation tube (3000 rpm, 2 min). Volume changes in both eluent and extraction solvent were then determined. Because it is not possible to inject the liquid scintillator or *o*-xylene into the LC system, the extraction efficiencies were calculated from the percentages of analytes, remaining in the aqueous phase by injecting an aliquot of this phase into the liquid chromatograph and comparing the UV peak heights with standard injections. The peak heights were corrected for the volume changes in the aqueous phase after extraction. Correction factors became important with increasing concentration of organic modifier in the eluent.

For determining the recovery of M1, the liquid scintillator could not be used because an unknown component extracted from the scintillator into the water phase co-eluted with M1. Therefore, *o*-xylene was used as a comparable extraction solvent.

Plasma sample preparation

After thawing and ultrasonification of the sample, a few milligrams of Na_2EDTA and a known amount of the internal standard were added to 1.0 ml plasma. The solution was extracted with 2 × 5 ml dichloromethane–propan-2-ol (9:1, %v/v) (i.e. at pH = 7). After centrifugation, the combined organic layers were evaporated to near dryness under a mild stream of nitrogen at 40°C. The solution was transferred to a tapered sample vial with the aid of absolute ethanol, and evaporated to dryness with nitrogen at 40°C. The residue was dissolved in 50 μl eluent (containing Na_2EDTA), of which 45 μl was injected by using the partial filling mode of the injector.

The exact volume injected can be calculated from the ^{14}C-signal of the internal standard. In this way, samples were injected 40–50 min after thawing. Na_2EDTA was added to prevent the rapid decomposition of metabolites during the sample preparation step, which is especially pronounced for the M1 metabolite. The effect of Na_2EDTA on the stability of the analytes has been studied in [6].

Preparation of the liquid scintillator solution

From batch experiments, it was found that dodecylbenzenesulphonate (DBS) was the most effective ion-pair agent. The DBS/scintillator solution was prepared as follows: 1.0 g DBS (2 mmol) was suspended in 20.0 ml *n*-butanol and after centrifugation, the clear, light yellow supernatant was decanted. The weight of the dried residue was 0.4 g. From these figures, the DBS concentration in *n*-butanol could be calculated to be 0.09 M. By pipetting known volumes of this solution into the scintillator, the DBS concentration in the scintillator can be determined.

Results and Discussion

Chromatography

Recently, an LC method has been published for the quantitation of urapidil and its metabolites in biological fluids by a direct injection and column-switching technique [7]. Separation was achieved on a 5-μm Nucleosil C-18 column (125 × 4.6 mm) at 40°C, using a methanol–water gradient in 20 mM sodium perchlorate (pH = 2.0) with electrochemical detection. The use of methanol, however, is not favourable for the segmentation of the eluent with the water-immiscible scintillator [4]. In addition, with the present set-up, gradient elution is not possible. Therefore, an isocratic LC system has been developed that produces a complete separation of the analytes and internal standard (see Fig. 3). The absorbance wavelength was optimized for urapidil (269 nm) although for remoxipride, sensitivity is maximum at 233 nm. However, because of the availability of [^{14}C]-remoxipride, it was still useful as an internal standard. It should be noted that remoxipride was used for calculating the volume recovery in the plasma sample preparation step only.

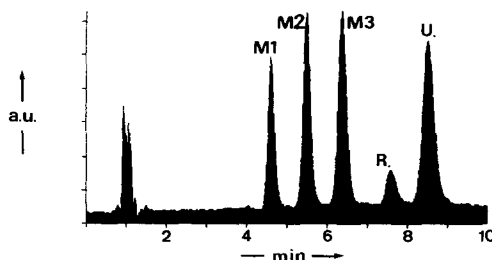


Figure 3
Chromatogram with UV detection (269 nm). Sample, urapidil (14.3 μM), M1 (9.3 μM), M2 (11.7 μM), M3 (13.4 μM) and remoxipride (71.2 μM); column, Brownlee cyano Spheri-5 (100 × 4.6 mm); eluent, acetonitrile–aqueous H_3PO_4 (pH 2.2), containing 1.0 mM sodium hexylsulphonate, (12:88, %v/v) at 1.0 ml min^{-1}.

Batch and on-line extraction

Extraction of radiolabelled analytes from eluent into the water-immiscible liquid scintillator is of crucial importance for the radioactivity detection as the mean range of the beta-particles of ^{14}C in water is about 30 μm [9], while the mean length of the water and scintillator segments varies between 2 and 20 mm.

For amine-type pharmaceuticals, extraction is a function of the pH of the aqueous phase and generally the optimum pH is about 9–10 [10]. In Table 1, the extraction percentages are given for pH = 9.6, as a function of the extraction solvent composition. In the presence of scintillator, no conditions were found that allowed the quantitative extraction of all analytes. In addition, at pH > 10 extraction of the metabolites decreased with increasing pH. This was especially pronounced for M1 and can be explained by phenolate formation of the metabolites. Therefore, the possibility of ion-pair extraction of the protonated analytes was investigated. Various ion-pair reagents were tested for their extraction efficiencies. The reagent was diluted in the eluent in excess over the total amount of analytes. From Table 2, it can be concluded that DBS is best suited as ion-pair reagent. Moreover, as compared to DDS, at equal concentration (<0.5 mM), phase separation was faster for the DBS solution. For the on-line experiments, DBS was added to the scintillator. In practice, 10 ml of the DBS/*n*-butanol solution in 1000 ml scintillator was satisfactory, both with respect to the segmentation process and the extraction percentages (which were found to be over 99%; see for example Fig. 4). This was also confirmed for M1 with DBS/*o*-xylene as extraction solvent

Table 1
The influence of extraction solvent composition on the extraction of urapidil, M1, M2, M3 and remoxipride from an equal volume of eluent*

Extraction solvent		Extraction percentage Ura.	M1	M2	M3	Rem.
Chloroform		>99	>99	>99	>99	>99
Scintillator		64	—	34	10	94
Scintillator/chloroform	(10/1, v/v)	84	—	7	22	93
Scintillator/butylacetate	(10/1, v/v)	69	—	39	6	93
Scintillator/*n*-butanol	(10/1, v/v)	88	—	69	48	94
Scintillator/*n*-octanol	(10/1, v/v)	90	—	75	47	95
Scintillator/*n*-octanol	(10/2, v/v)	96	—	86	71	95
Scintillator/*n*-octanol	(10/12, v/v)	98	—	94	86	97
n-Octanol		95	84	89	80	99
O-xylene		77	4	49	6	97

*The extraction percentages are calculated from the percentage remaining in the water phase, assuming 100% recovery. Eluent acetonitrile–aqueous carbonate buffer (pH 9.6), (10:90, % v/v); scintillator, Ready Solv NA.

Table 2
Ion-pair extraction of urapidil, M1, M2, M3 and remoxipride from eluent into chloroform (1:1, v/v)*

Ion-pair reagent	Excess†	Extraction percentages Ura.	M1	M2	M3	Rem.
DAS	12	97	7	62	60	>99
Naphthalene-2-sulphonate	17	65	14	20	16	88
Hexylsulphonate	20	70	6	30	17	93
Dodecylsulphonate	19	>99	>99	>99	>99	>99
DBS	6	>99	>99	>99	>99	>99

*Extraction percentages are calculated from percentage remaining in the water phase, assuming 100% recovery. Eluent, acetonitrile–aqueous H_3PO_4 (pH 2.2), (5:95, % v/v).
†Excess of ion-pair reagent with respect to the total amount of analytes.

under otherwise identical conditions. Under these circumstances, the DBS concentration in the extraction solvent was calculated to be 1 mM.

Influence of eluent composition

Normally, the only parameter in the eluent composition to be varied is the percentage of organic modifier. Consequently, the percentages of urapidil, M2 and M3 extracted into the DBS/scintillator and the corresponding distribution coefficients, D, were determined, as a function of the percentage acetonitrile in the eluent. Correction has to be made for the volume changes in the aqueous phase. These changes can be ascribed to solution of acetonitrile from the eluent into the organic phase [4]. Simultaneously, the counting efficiency in the scintillator after extraction and phase separation was determined. This was accomplished by addition of known amounts of [^{14}C]-urapidil to the scintillator phase and off-line counting. The results are plotted in Fig. 4. For acetonitrile contents of less than 20%, the extraction of the analytes was almost quantitative with only a slight decrease in counting efficiency. So far, it has not been possible to explain the distribution curves fully. They probably result from a competition between the dissociation of the ion-pairs in the scintillator phase followed by back extraction of the ion-pair reagent and the free molecules [11]. Also, the possibility of micelle formation of DBS, extracted from the scintillator into the aqueous phaes, should

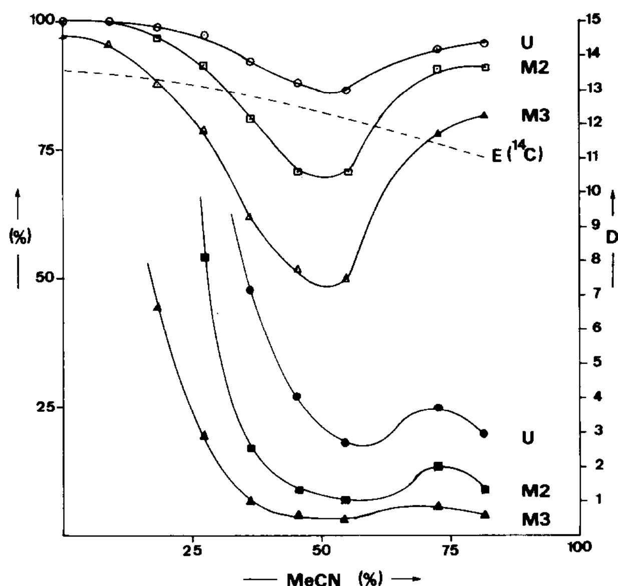


Figure 4
Extraction percentages (open symbols) and distribution coefficients (black symbols) of urapidil, M2 and M3 as ion-pairs with DBS from eluent (pH = 2.2) into liquid scintillator (Ready Solv Na, 1:1 v/v) as a function of the percentage acetonitrile in the eluent. The dashed curve gives the ^{14}C-counting efficiency of the scintillator after phase separation. The distribution coefficients are calculated from $[C]_{org}/[C]_{aq}$. For explanation: see text.

be considered. Below a certain volume, the DBS concentration in the water phase may reach its critical micellar concentration (CMC), which is reported to be 2 mM [12]. This effect may take place at high acetonitrile contents.

Direct measurement of [^{14}C]-urapidil

The effect of ion-pairing on extraction and, thus, the counting efficiency is demonstrated in Fig. 5B, C. [^{14}C]-urapidil was injected twice, without and with DBS in the scintillator, respectively, at F(eluent) = 1.0 ml min^{-1} and F(scintillator) = 0.25 ml min^{-1}. The counting efficiencies calculated from equation (1) are 0.03 and 0.48, respectively. By increasing the F(scintillator)/F(eluent) ratio, E values of over 0.9 were obtained. However, when the total flow-rate through the radioactivity monitor increases, the counting time T_d decreases. Thus, a compromise between an optimum E value [determined by F(scintillator)/F(eluent)] and optimum T_d (determined by the total flow-rate) has to be found. This is illustrated in Fig. 6. [^{14}C]-urapidil was injected repeatedly with F(eluent) = 1.0 ml min^{-1}, and the ^{14}C-peak area was recorded as function of F(scintillator). For this system, at F(scintillator) = 0.5 ml min^{-1}, an optimum was found. Even for F(scintillator)/F(eluent) = 0.2, the E value and the reproducibility of ^{14}C-determination were still satisfactory; at this ratio, RSD = 2.2% (3400 Bq injected, 1.1 ml extraction coil, N = 10) with E = 0.68. With F(eluent) = F(scintillator) = 1.0 ml min^{-1}, good linearity was found between the net peak area C_n (in counts) and the amount [^{14}C]-urapidil injected (in DPM), i.e. $C_n = 80.5 \times \text{DPM} - 14.6$ (N = 15, r = 0.9999, $2 \times 10^3 < \text{DPM} < 180 \times 10^3$).

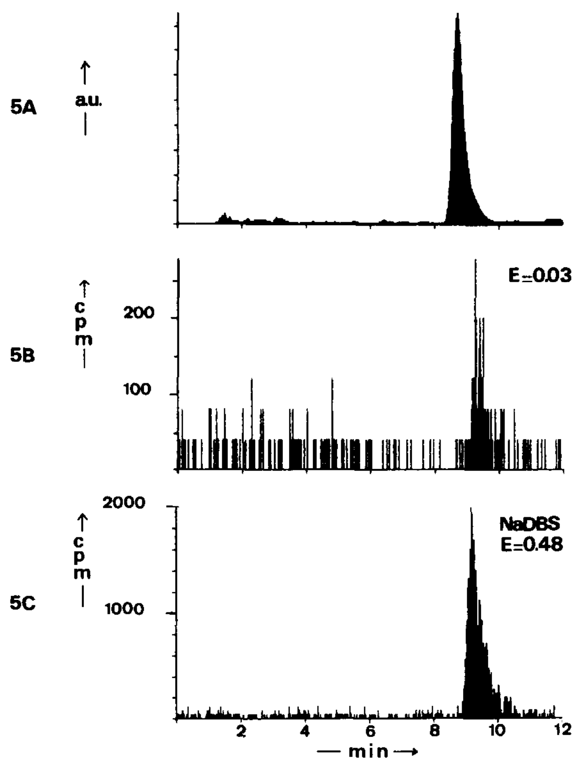


Figure 5
UV (5A) and on-line radiometric detection of [^{14}C]-urapidil without (5B) and with (5C) ion-pair reagent in the scintillator. Sample, 730 Bq (0.72 nmol) [^{14}C]-urapidil in 23 μl eluent; scintillator, Ready Solv NA (5B) or Ready Solv NA containing 2.0% v/v *n*-butanol/DBS (5C); *F*(scintillator) = 0.25 ml min^{-1}; 0.42 ml extraction coil. For other conditions; see Fig. 3.

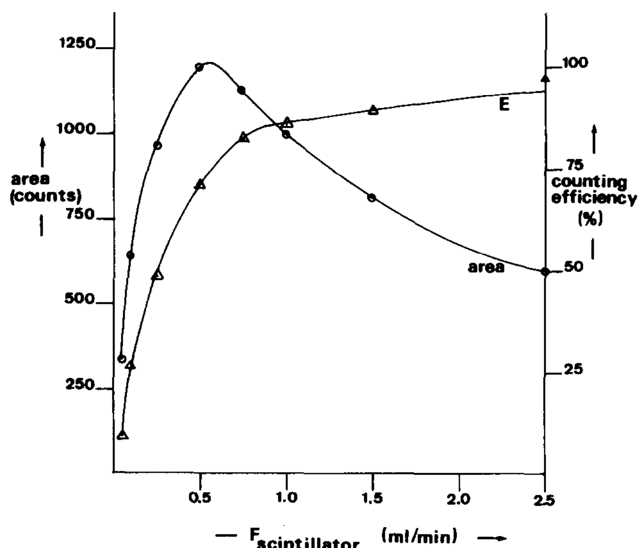


Figure 6
On-line ^{14}C-peak area and ^{14}C-counting efficiency as a function of *F*(scintillator). Sample, 730 Bq (0.72 nmol) [^{14}C]-urapidil in 23 μl eluent; scintillator, Ready Solv NA containing 2.0% (v/v) *n*-butanol/DBS. Other LC conditions, see Fig. 3.

Reverse measurement of [^{14}C]-urapidil

An example of a direct and corresponding reverse radiogram is given in Fig. 7. In the reverse radiogram, two so-called "regions of interest" were used: during the transport of [^{14}C]-urapidil and the dead volume of the chromatogram through the monitor, the flow-rate was 75 μl min^{-1}. The rest of the chromatogram was pumped at 1.0 ml min^{-1}. The dead volume is sampled for an accurate background determination because, in principle, no activity elutes in this zone. From the flow-rates and the effective flow-cell volume, counting times of 0.83 and 0.03 min were calculated, respectively. The effect of the counting time on the ^{14}C-peak area is clearly seen. In this way, the influence of T_d on the ^{14}C-peak was studied. Repetitive injections of [^{14}C]-urapidil (35 Bq, 33 pmol) were made while varying T_d (by varying the flow-rate F_t) in the reverse measurements. The linearity of the net peak area with $0.5 < T_d < 210$ (s) was satisfactory ($r = 0.999$, $N = 15$), although care must be taken in the use of the effective flow-cell volume V_d for calculation of T_d because V_d has been shown to depend on the flow-rate through the radioactivity monitor [13]. The corresponding flow-rates were determined by collecting the volume pumped during the regions of interest.

By comparing direct radiograms with corresponding reverse radiograms, it was found that band broadening during storage and transfer of [^{14}C]-urapidil was negligible, even for counting times of over 1 min (corresponding to a reverse measuring time of 300 min; data not shown).

Analysis of plasma samples

In radioactivity determination, the counting time needed will depend on the amount of activity injected. In order to keep the analysis time within reasonable limits, the amount

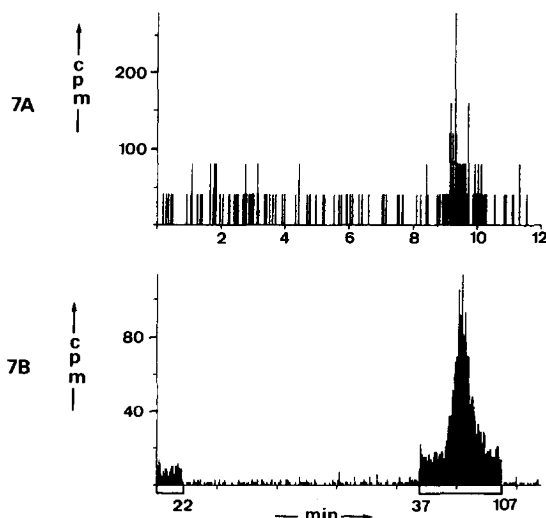


Figure 7
Direct (7A) and corresponding reverse (7B) measurement of [^{14}C]-urapidil, using "regions of interest" in the reverse measurement. Sample, 35 Bq (0.35 pmol) [^{14}C]-urapidil in 23 μl eluent; for the direct measurement, $F(s) = F(e) = 1.0$ ml min^{-1}; total flow-rate = 2.0 ml min^{-1} ($T_d = 0.03$ min); for the reverse measurement, $F(e) = 75$ μl min^{-1} during the "regions of interest" ($T_d = 0.83$ min); the rest of the chromatogram is pumped at $F(e) = 1.0$ ml min^{-1} ($T_d = 0.06$ min) [$F(s) = 0.0$ ml min^{-1}]. In Fig. 7B. The regions of interest are indicated by brackets on the time axis. Other LC conditions, see Fig. 6.

of activity injected in the chromatograph should be as high as possible. This was accomplished by using the partial filling mode of the injector. In that way, 90% of 1.0 ml plasma samples could be injected after their concentration into 50 μl of eluent. The reproducibility of this injection method was acceptable (RSD = 5 and 3% for radioactivity and UV detection, respectively, N = 10). The UV chromatogram and the on-line radiograms (direct and reverse) were compared with off-line radioactivity determination. For the latter, after UV detection, fractions were collected which contain in the total peak elution volumes of urapidil and its metabolites. An example is given in Fig. 8 with UV, direct and reverse radiogram. The percentage radioactivity remaining in the aqueous phase after sample preparation was 16.7%. In this experiment, the internal standard could not be used because of partial co-elution with M3. This was caused by the excessive use of the column before measuring the plasma samples, which resulted in a decrease of the column performance, i.e. for urapidil the column efficiency dropped from N = 4200 to N = 2000 theoretical plates after 4 weeks use.

From a comparison with direct radiograms of [^{14}C]-urapidil standards (recorded under identical conditions), it was evident that the present tailing of the urapidil peak was possibly caused by an impurity in the administered [^{14}C]-urapidil. In Table 3 the percentages of metabolite in rat plasma measured by four detection methods are given.

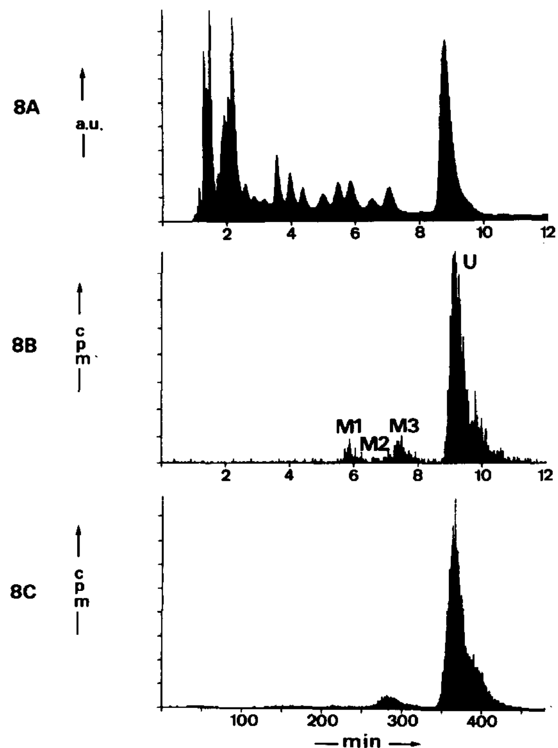


Figure 8
UV chromatogram (8A) and corresponding direct and reverse radiograms (8B and C, respectively) of rat plasma (taken 60 min after administration of [^{14}C]-urapidil). Direct measurement: F(e) = F(s) = 1.0 ml min^{-1}; (T_d = 0.03 min); reverse measurement: F(e) = 52.7 μl min^{-1} (T_d = 1.34 min). For sample preparation, see text. For other LC conditions, see Fig. 6.

Table 3
Percentage metabolite (relative to urapidil added) in rat plasma, calculated from peak areas in the UV chromatogram and the direct and reverse radiograms, given in Fig. 8A, B and C respectively, compared to off-line radioactivity determination. For UV data, no RSD values were determined (see text)

	M1 (%)	M2 (%)	M3 (%)
UV	6.3	4.3	7.5
Direct radiogram	4.5 ± 0.5	2.1 ± 0.4	6.7 ± 0.7
Reverse radiogram	0.3 ± 0.2	<0.1	6.3 ± 0.2
Off-line determination	7.8 ± 0.2	2.1 ± 0.1	9.5 ± 0.2

Data calculated from the UV chromatogram are not considered to be accurate due to the small signals from the metabolites, the partial overlap of M1 with an unknown compound from the plasma sample and the tailing of peaks with a fluctuating background signal. RSD values for the UV peak areas could not be determined. The RSD values given for the radioactivity detection modes were calculated from statistical fluctuations in the ^{14}C-signals and background only. In practice, for off-line counting, the actual RSD values may be larger because of the uncertainty in the volume sampling for closely eluting peaks.

From Table 3, the lower percentage of M1 and M3, calculated from the direct radiogram compared to off-line counting can be ascribed to the differences in the extraction percentages of urapidil, M1 and M3 during the direct measurement (see for example Fig. 4). For the extraction coil used, the ratio *F*(scintillator)/*F*(eluent) was too low to give quantitative extraction of all analytes. This explanation is confirmed by comparing the percentage of M2 formed, calculated from the direct measurement and off-line counting. From Fig. 4 the extraction properties of urapidil and M2 are expected to be identical for an acetonitrile percentage of less than 10%. In this case, the ratio of the ^{14}C-peak areas for M2 and urapidil will give the true percentage of M2 formed and should be identical to the percentage calculated from the off-line detection. This is found to be the case.

The quantitation of the metabolites in biological fluids is hampered by their instability in these fluids at room temperature [6]. If degradation products are assumed to be less extractable, this probably explains the disappearance of M1 and M2 from the reverse radiogram. A possible solution to this problem may be the addition of Na_2EDTA to the eluent. In a separate experiment it was found that the addition of 1.0 mM Na_2EDTA to the eluent did not adversely affect the LC separation or the extraction percentages of urapidil, M2 and M3 (M1 was not included). However, the use of Na_2EDTA in the eluent led to excessive pressure build-up, probably caused by complexation and precipitation on the 2 μm frits used in the LC system. Consequently, this was not explored any further.

Conclusions

Urapidil, its main metabolites and the internal standard remoxipride can be quantitatively extracted from an aqueous phase of pH = 2.2, containing 15% (v/v) acetonitrile into a water-immiscible liquid scintillator using ion-pair extraction with sodium dodecylbenzenesulphonate (DBS). It is advantageous to dissolve the ion-pair

reagent in the scintillator so that no extra reagent pump is needed. Addition of DBS to the eluent would give rise to an increase in the UV background absorbance and, consequently, to a decrease in the UV sensitivity.

The post-column extraction and segmentation principle enables the storage of the complete chromatogram, and the use of prolonged counting times in "regions of interest" in reverse measurements. A linear relationship was found between the net ^{14}C-peak area (as determined in reverse measurements) and the counting time T_d while band broadening during storage was minimal even for counting times of over 1 min. Unfortunately, due to the instability of the metabolites, reverse measurements could not be used in the application of the principle to plasma samples. Under these circumstances, only direct measurements will give accurate information on the metabolism pattern. The direct injection method (see [7]) should be preferred because it prevents some of the difficulties frequently encountered in liquid–liquid extraction such as poor recovery, formation of artefacts and reduced stability of the metabolites.

From the influence of the ratio F(scintillator)/F(eluent) on the extraction efficiency and, thus, on the counting efficiency of the radiolabelled analytes, it follows that (as with all methods of analysis based on post-column extraction techniques) the calculation of the percentages of metabolites formed (relative to urapidil) from the on-line radiogram is possible only if at least one of the following conditions is fulfilled. (1). The individual on-line extraction efficiencies are known. This requires the use of ^{14}C-labeled standards of urapidil and its metabolites. In this study, only ^{14}C-labeled urapidil was available. (2). If for all analytes equilibrium in on-line extraction is reached, the percentage of metabolites formed can be calculated using correction factors that can be taken from the extraction percentages determined in batch experiments with non-labelled standards. The conditions for which equilibrium is reached can be found by repetitive injection of an aliquot of plasma (with ^{14}C-labelled metabolites) while varying the length of the extraction capillary [14]. (3). The extraction percentages for all analytes are over 90%. For this system, at F(eluent) = 1.0 ml min^{-1}, this will probably hold for F(scintillator) >3 ml min^{-1}.

If condition (1) or (2) is fulfilled, F(scintillator)/F(eluent) ratios down to 0.2 can be used in the direct measurement. Although in that case the counting efficiency is not optimal, the sensitivity and the reproducibility of ^{14}C-detection are still satisfactory, due to the corresponding increase in the counting time. The figures compare favourably with the use of a water-miscible liquid scintillator in on-line radioactivity detection. Generally, in that case, F(scintillator/F(eluent) ratios of over 3 must be used [15] with a corresponding increase in the cost of scintillator and disposal of radioactive waste.

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