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QUANTITATIVE ANALYSIS OF PRAZEPAM AND ITS METABOLITES BY ELECTRON CAPTURE GAS CHROMATOGRAPHY AND SELECTED ION MONITORING

APPLICATION TO DIAPLACENTAL PASSAGE AND FETAL HEPATIC METABOLISM IN EARLY HUMAN PREGNANCY

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SUMMARY

Methods have been developed for the determination of the benzodiazepine tranquilizer prazepam and its metabolites desmethyl diazepam, 3-hydroxy-prazepam and oxazepam by electron capture gas chromatography and selected ion monitoring with diazepam as the internal standard. The benzodiazepines were isolated from blood serum or homogenized tissue samples, either by extraction with ethyl acetate or on small Extrelut columns packed with porous silica. The concentrated extracts were directly injected into the gas chromatograph equipped with an electron capture detector. Following trimethylsilylation, analysis on a gas chromatography—mass spectrometry—computer system operated in the selected ion-monitoring mode was performed. Using 50—200 mg (μl) biological material, concentrations of prazepam and metabolites of 5 ng/g(ml) could be determined with signal-to-noise ratios of >10. Using 1 g(ml) samples, the same signal-to-noise ratios were obtained with 1 ng/g(ml) concentrations.

The methods developed were applied to the analysis of the diaplacental transfer of prazepam and desmethyl diazepam in early human pregnancy. Furthermore, prazepam metabolism in human fetal liver and cell cultures was studied.

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INTRODUCTION

It has been recognized in recent years that almost all foreign substances taken in by the mother including drugs, environmental contaminants, food additives, the constituents of cigarette smoke, alcohol, etc. cross the placenta, enter the fetal blood stream and may be stored in fetal tissues [1, 2]. It has also been demonstrated that from a relatively early stage (6—8 weeks of gestation) the human fetus can metabolize a variety of foreign compounds [1, 3—5].

While metabolism can lead to detoxification of harmful substances, it may also result in the production of toxic or pharmacologically active metabolites from otherwise innocuous compounds. Frequently such derivatives are more polar than the parent compounds and slowly cross the placenta back to the mother. Such transfer characteristics may result in an accumulation of metabolites in the fetus. It is therefore important to determine the concentrations of metabolites as well as those of the parent compounds in the fetus. In contrast to man, common laboratory animal species do not possess the ability to metabolize xenobiotics at such an early stage of gestation. Therefore, results obtained from studies with animals cannot be reliably extrapolated to the human situation and may underestimate the risk at hand, thus making studies on human subjects essential.

Of particular interest is the transfer of xenobiotics from mother to embryo during organogenesis (4—8 weeks of gestation) at which time the conceptus is most sensitive to teratological lesions. Although fetal tissue is available from therapeutic abortions during the first trimester, the amounts of clearly identifiable material obtained at this early stage of human gestation are often only in the milligram range. Therefore, analytical methods with high sensitivity are required to investigate such material.

This report describes analytical methods suitable for the study of the diaplacental transfer of prazepam (a benzodiazepine) as well as its metabolites during early human pregnancy and for in vitro investigations of the metabolism of prazepam in cultures of human fetal liver. A gas chromatograph equipped with an electron capture detector and a GC—MS—computer system used in the selected ion monitoring mode are shown to provide sufficient sensitivity and selectivity to achieve the experimental goals. Several results are reported as illustrative examples of the suitability of the analytical methods.

EXPERIMENTAL PROCEDURES

Solvents and reagents

Extrelut and pyridine ("getrocknet") were obtained from Merck (Darmstadt, G.F.R.), ethyl acetate, benzene and methanol (nanograde) from Byk-Mallinckrodt (Wesel, G.F.R.), Tri-Sil BSA formula P from Pierce (Rotterdam, The Netherlands), the GC column-packing material 3% SP-2250 on Supelcoport 100—120 mesh from Supelco (Bellefonte, Pa., U.S.A.).

Internal standard

A stock solution of 6 mg diazepam in 10 ml of methanol was prepared and

diluted 1:10 with methanol prior to storage in 1.5 ml glass vials at —25°; 10.0 µl of this dilution (= 60 ng diazepam) were added to each sample as early as possible (see below). We have used identical amounts of methyl-d_3-diazepam as internal standard for the analysis of the metabolites of various benzodiazepine drugs including medazepam (Nobrium) and diazepam (Valium), prazepam (Demetrin) by selected ion monitoring, hence the label with deuterium was necessary.

Standard calibration graphs were obtained by adding known amounts of prazepam and its metabolites (between 2 and 200 ng) to 200-µl portions of drug-free human serum containing the internal standard (60 ng); see Figs. 1 and 2. A linear least-square analysis of the peak height ratios vs. amounts of drug and metabolites added was made.

Biological material

Human fetuses (between 6 and 24 weeks of gestation) were obtained by hysterotomy, curettage, or prostaglandin-induced abortions for social or medical reasons. Maternal blood samples were taken at the time of the interruptions. For studies of the diaplacental passage of prazepam, the fetuses were kept frozen on dry ice until dissection began. The fetal material was allowed to thaw and the various tissues were carefully dissected and stored in 1.5 ml conical plastic semi-micro tubes (Eppendorf) at —25°. At the time of analysis, 10—200 mg of the frozen tissues, depending on availability, were weighed and transferred into Eppendorf tubes. A volume of 200 µl of deionized water and 60 ng internal standard were added and the samples were homogenized either with a conical PTFE pestle made to fit the conical tubes or by sonication at 0°. Both methods gave identical results.

For the preparation of fetal liver organ cultures and of isolated fetal liver cells, the livers were excised from the fetuses as soon as possible and then pro-

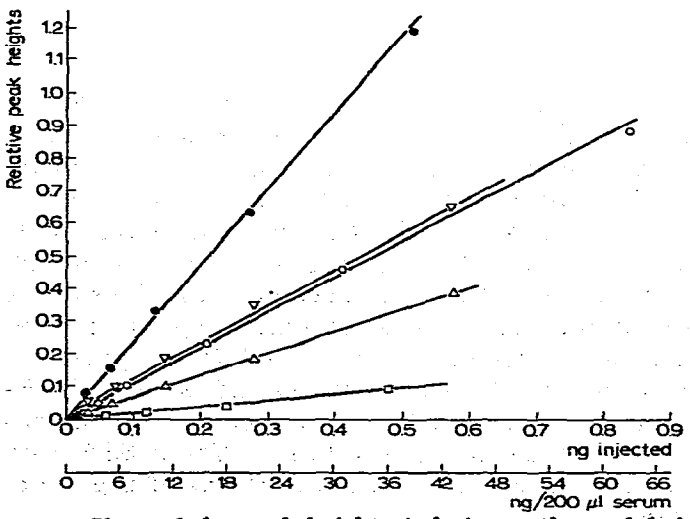


Fig. 1. Plots of the peak heights (relative to the peak height of diazepam, internal standard) of Pr and metabolites vs. amounts present in 200 µl serum as well as in injected samples (detection by electron capture GC). •, Pr-OH (TMS); ▽, Ox; ○, DD; △, Pr; □, Pr-OH.

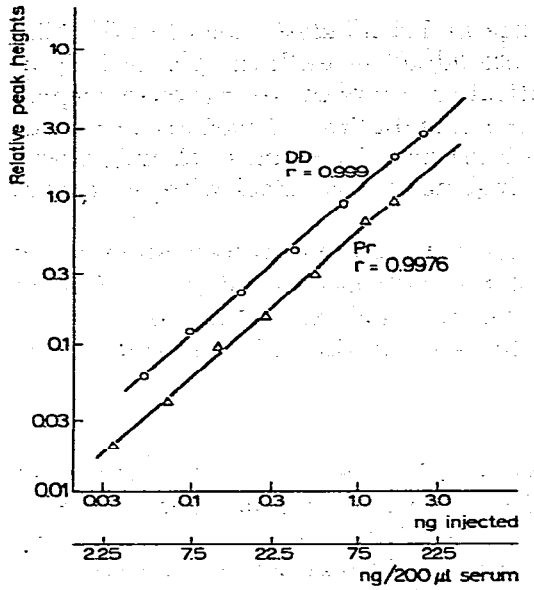


Fig. 2. Double logarithmic plots of the peak heights of Pr and DD (relative to the internal standard, diazepam) vs amounts present in 200 µl serum as well as injected samples (detection by electron capture GC).

cessed as described elsewhere in detail [6]. Prazepam, 0.5—20 µg per ml incubation medium, was added to the final suspension of the fetal tissue and the cultures were incubated on a gyratory shaker (60 gyrations per min) in an atmosphere of 95% O_2 /5% CO_2 at 37°. Samples were removed at selected time intervals, sonicated and frozen. At the time of analysis, 200 µl of the homogenized samples were transferred into 1.5 ml Eppendorf tubes and 60 ng of internal standard was added.

Extraction

Three methods for the isolation of prazepam and metabolites were explored:

(a) To 200 µl of the various samples to be analyzed (e.g. maternal serum, fetal tissue homogenates or homogenates of fetal liver cells or organ culture material, all of which contained 60 ng of internal standard), 1 ml of ethyl acetate was added. The Eppendorf tubes were tightly closed, shaken on a Vortex mixer for 2 min and centrifuged at 550 *g* for 2 min. A volume of 800 µl of the organic phases were transferred into 1.5-ml glass vials and the solvent was evaporated under a stream of nitrogen. Traces of remaining water were removed by the addition of 100 µl benzene and a repeat of the evaporation. Finally, 30 µl of pyridine were added, the vials were closed with a PTFE-lined rubber septum using a hand crimper, and 1 µl was injected into the gas chromatograph. If the samples were to be silylated, 30 µl of Tri-Sil BSA were added, and the vials were incubated at 55° for 30 min. Portions of 2 µl were injected into the gas chromatograph.

(b) The 200 µl samples to be analyzed were diluted with up to 200 µl of deionized water and poured into Pasteur pipettes (Fig. 3) filled with a weighed

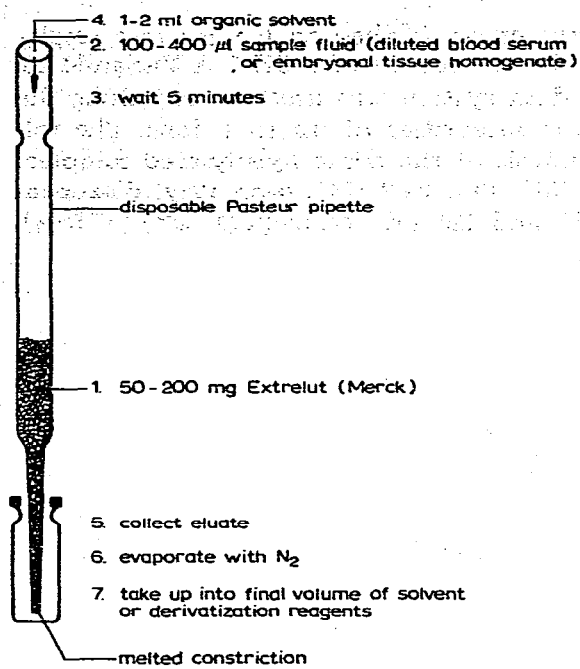


Fig. 3. Schematic representation of the isolation of benzodiazepines and their metabolites from small samples (5—200 mg) of human embryonal and fetal tissue (following homogenization) and 50—200 µl of serum or plasma (following dilution by factor of 2).

amount of Extrelut [7] in a proportion of 50 mg Extrelut for each 100-µl sample volume. After 5 min the columns were eluted with 1—2 ml of ethyl acetate, the eluate collected in 1.5-ml glass vials and further processed as described under (a).

(c) To the 200-µl samples, 100 µl of butyl acetate [8] were added and the contents of the tubes were Vortex mixed for 2 min. Following centrifugation, 2.5 µl of the supernatant butyl acetate phase were carefully withdrawn into a 10-µl syringe and injected into the gas chromatograph.

Gas chromatography

A Carlo-Erba 2300 gas chromatograph equipped with an electron capture detector was used. The detector (HT-20) was operated in constant current mode (Model 250). A 2-m glass column was used which was packed with 3% SP-2250 on Supelcoport 100—120 mesh. The oven temperature was kept at 260° for the simultaneous analysis of all prazepam (Pr) metabolites and at 280° for the determination of the trimethylsilylated 3-hydroxy-prazepam (Pr-OH TMS). The temperature of the injection port was 275° and of the detector 300°. Nitrogen (Linde, Munich, G.F.R.; purity 5.0) was used as carrier gas (20 ml/min) and scavenger gas between column and detector (40 ml/min).

Selected ion monitoring on a GC—MS—computer system

This system consisted of a Perkin-Elmer F-22 gas chromatograph which was

directly coupled via a 1/4 in. Swagelok union (drilled to 6 mm) to a single-stage Watson—Biemann separator and the ion source of a CH 7 A Varian-MAT mass spectrometer. A Varian SS-100 data system was used for switching the acceleration voltage and monitoring the intensities of up to 8 ions. The following ions were recorded for the analysis of the trimethylsilylated samples: *m/e* 287(M^+, internal standard); 324 (M^+, Pr); 342 (M^+, desmethyl diazepam (DD) TMS); 383 (M^+—29, Pr-OH TMS); and 429 (M^+, oxazepam (Ox) $(TMS)_2$). Calibration graphs are shown in Fig. 4.

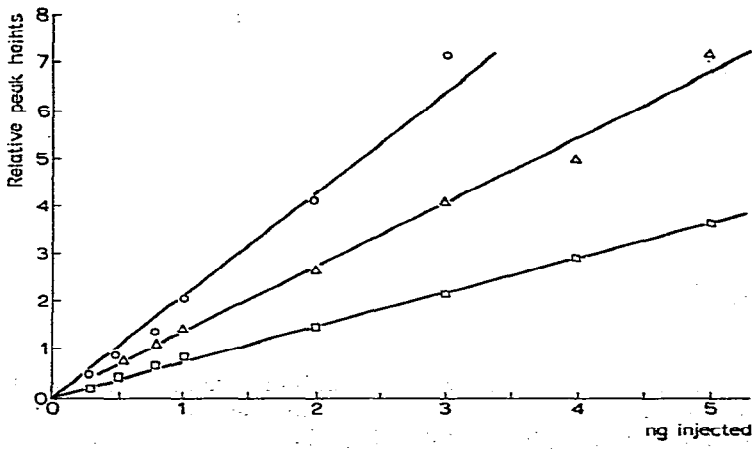


Fig. 4. Plots of peak heights of ions corresponding to Pr and metabolites (relative to ion *m/e* 287, methyl d_3-diazepam used as i.s.) vs. amounts present in injected samples (detection by selection ion monitoring). ○, DD (trimethylsilylated), r = 0.985; △, Pr, r = 0.9987; □, Pr-OH (trimethylsilylated), r = 0.9994.

RESULTS

Isolation Procedures

Three methods for the isolation of prazepam and metabolites from biological material have been used (see Experimental). The yields of the benzodiazepines studied obtained by method (a) (single-step ethyl acetate extraction) and method (b) (using small Extrelut columns) are compared in Table I. Higher yields were obtained with procedure (a) with the exception of Pr-OH whose recovery was higher with procedure (b). A comparison of the electron capture gas chromatograms of two samples which have been prepared with procedures (a) and (b) revealed that a lower baseline was obtained when the ethyl acetate extraction rather than the Extrelut column work up was used.

Method (c) was faster than either method (a) or (b) and resulted in high yields of all metabolites studied. However, the gas chromatographic base lines obtained are somewhat higher than those resulting from method (a). This method may be preferable if benzodiazepines are present in concentrations exceeding 50 ng/ml(g). Since high sensitivity was required in most of our experiments, we have routinely used method (a).

TABLE I

RECOVERY OF PRAZEPAM AND METABOLITES DURING ISOLATION PROCEDURES (a) AND (b).

Benzodiazepines	Recovery (%)	
	Ethyl acetate extraction*	Extrelut column**
Prazepam	93.5	80
Desmethyl diazepam	99.6	85
3-Hydroxy prazepam	83	93
Oxazepam	75	69
Diazepam (internal standard)	97.3	86

*Single-step ethyl acetate extraction.
**On small columns filled with porous silica (Extrelut).

Gas chromatography

Pr and its metabolites elute well-separated from each other and from the internal standard used (diazepam) on an SP-2250 column kept isothermally at 260° (Fig. 5). The detection limits of the compounds analyzed are presented in Table II. Pr-OH eluted relatively late (though as a well shaped peak) and therefore was not detected with sensitivity comparable to the other compounds. The volatility of this metabolite could be greatly enhanced by trimethysilylation, and the resulting TMS ether eluted conveniently just after Pr (Fig. 6). The lower limit of detection of this derivative was also comparable to Ox, DD, and Pr. Thus, if low levels of Pr-OH (<50 ng/g) were to be quantitated, the sample was first directly injected into the gas chromatograph to detect Ox, DD, and Pr (Fig. 6B). Then, the trimethylsilylating reagent was added and the resulting mixture injected for the quantitation of Pr-OH (Fig. 6C).

Selected ion monitoring

All metabolites of Pr could be quantitated in a single analysis using a GC—MS—computer system. The sample was trimethylsilylated and injected into the gas chromatograph. With the aid of the computer, the acceleration voltage of the mass spectrometer was switched rapidly to focus the molecular ions of the compounds to be analyzed sequentially at the electron multiplier detector. Since the molecular ions of the benzodiazepines studied are very abundant, high sensitivity was obtained which was comparable to the electron capture detector. The specificity of selected ion monitoring for the detection of benzodiazepines and their metabolites was even higher than electron capture GC, and all Pr metabolites could be quantitated in a single experiment either isothermally (Fig. 7B) or with linear temperature programming (Fig. 7A).

Calibration plots

Precisely measured amounts of Pr, DD and Pr-OH were added to 200-μl human serum samples in addition to 60 ng internal standard. The samples were then processed by the ethyl acetate extraction procedure (a) and analyzed

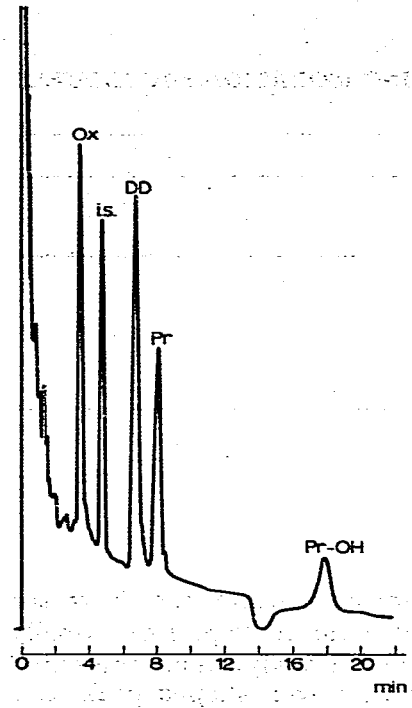


Fig. 5. Electron capture gas chromatogram (isotherm, 260° of a butyl acetate extract of human serum (extraction method, c; see Experimental); the injected sample contained 750 pg each of Ox, diazepam (internal standard), DD, Pr and Pr-OH.

TABLE II

LOWER DETECTION LIMITS AND RELATIVE STANDARD DEVIATIONS OF THE BENZODIAZEPINES DETERMINED BY ELECTRON CAPTURE GC

Signal-to-noise ratio = 2.

Benzodiazepines	Lower detection limit (pg)*	Relative standard deviation**	
		Day—day variation	Single-day variation
Diazepam	1.8	Internal standard	Internal standard
Prazepam	7.2	5.9	3.7
Desmethyl diazepam	4.1	4.2	2.8
Oxazepam	5.7	9.1	4.9
3-Hydroxy prazepam -TMS	29 1.5	7.5	5.9

*In terms of concentrations: 1—5 ng/ml serum or ng/g fetal tissue using 1000—50 μl (mg) biological materia[1].

**Determined in 200-μl human serum samples during one month; concentrations of the benzodiazepines between 2 and 40 ng/200 μl serum.

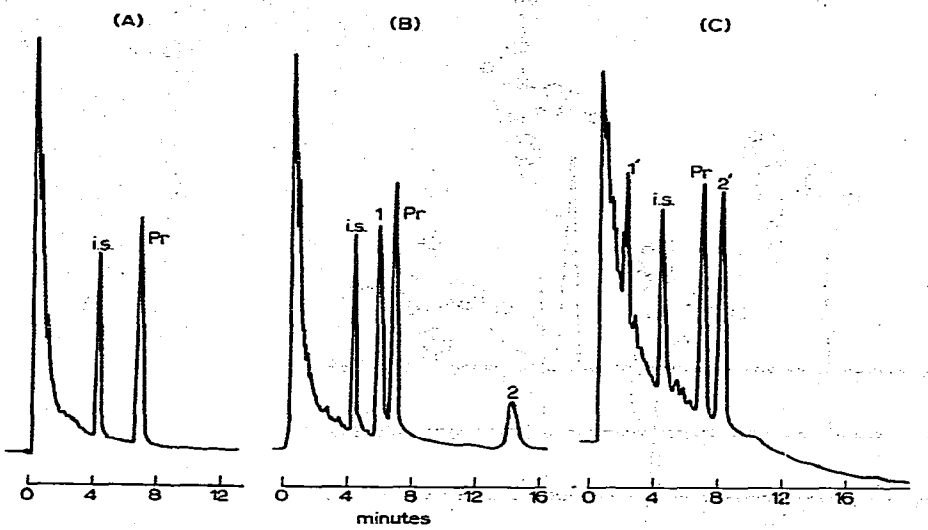


Fig. 6. Electron capture gas chromatograms of ethyl acetate extracts (extraction method a) of in vitro cultures containing Pr: (A) without liver; (B) with fetal liver (human fetus 10 weeks gestation), 1 = DD, 2 = Pr-OH; (C) sample B following trimethylsilylation, 1′ = DD, 2′ = Pr-OH, (both TMS).

by electron capture GC. Linear relationships between the relative peak heights referred to the internal standard vs. amounts added to the serum samples, were obtained for all compounds studied (Fig. 1). Double logarithmic plots of the relative peak heights of DD and Pr vs. amounts added, showed a linear range over more than three orders of magnitude. The plots covering two orders of magnitude, which we have used routinely for quantitation in our experiments, are shown in Fig. 2, where a slope of 1.0 indicates linearity in a double logarithmic presentation.

Comparable results were obtained on the GC—MS—computer system used in the selected ion monitoring mode (see Fig. 4).

DISCUSSION

The benzodiazepines are a thoroughly investigated class of drugs and a large selection of literature describing the analysis of these compounds exists [9, 10]. The analytical methods for Pr are, however, not as multifarious as those for many other benzodiazepines.

In pharmacokinetic studies involving Pr, DiCarlo and co-workers [11] made use of ^{14}C-ring-labelled Pr. The absolute level of activity in samples was measured by scintillation spectrometry. Pr and its metabolites were separated by thin-layer chromatography (TLC) and relative amounts were determined using a radioscanner.

Maier and Wehr [12] have reported a thin-layer and a GC method for the identification of Pr. The latter method relies upon the acid hydrolysis of Pr to the more volatile benzophenone. This method was, however, not suitable for our investigational goals since it did not allow a distinction between drug and metabolite(s).

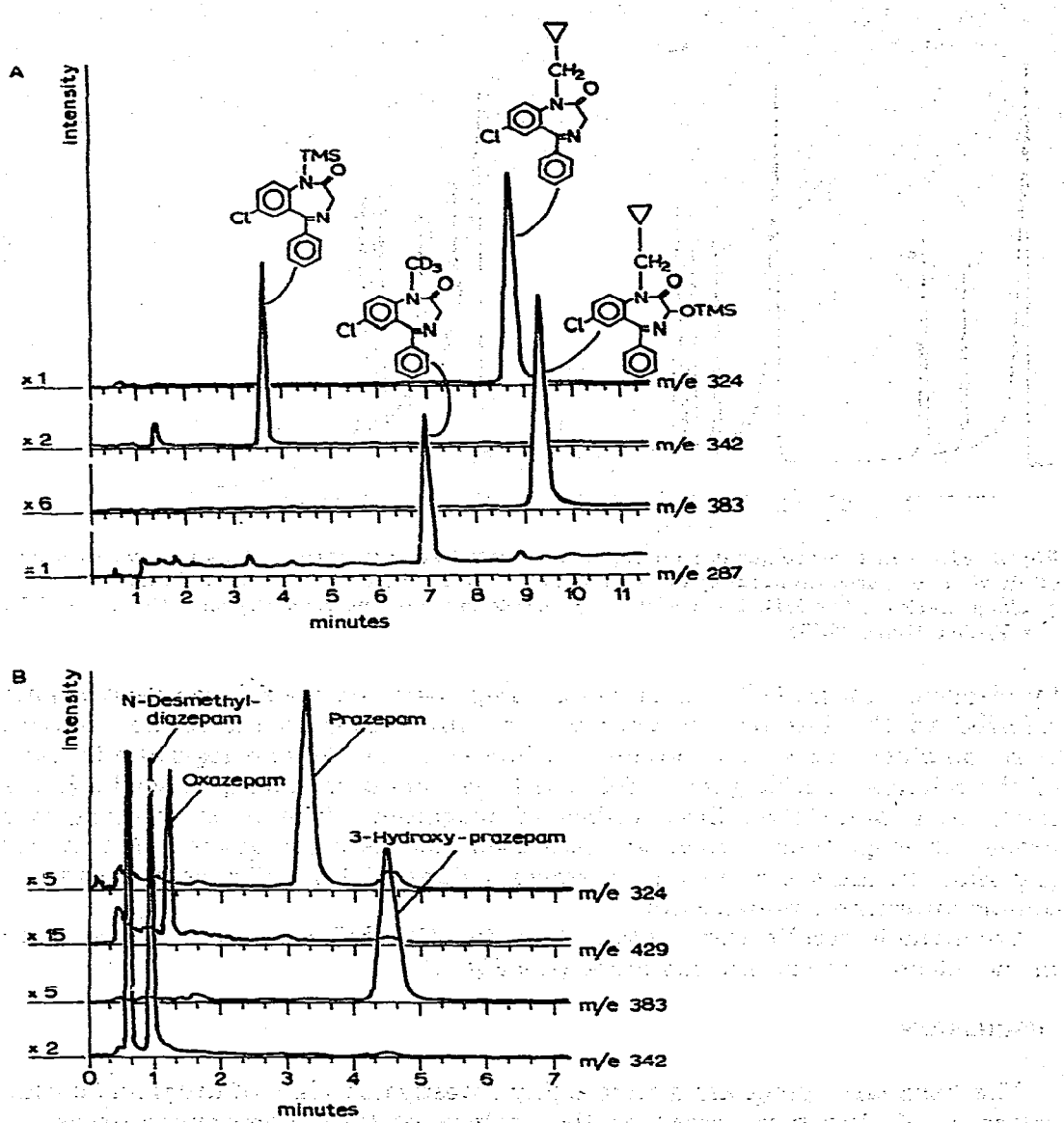


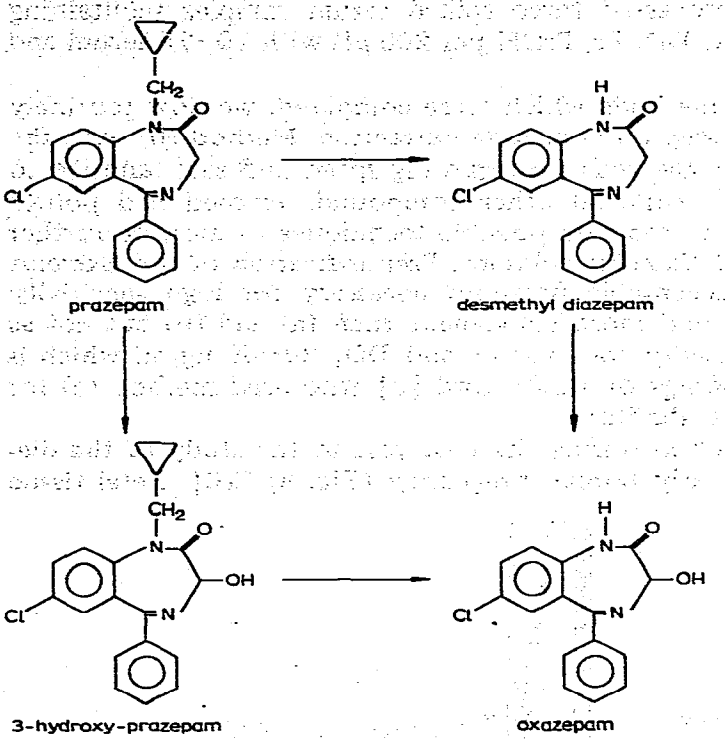
Fig. 7. (A) Selected ion monitoring (temperature program from 200 to 260° with 10°/min) of the trimethylsilylated ethyl acetate extract of Pr metabolites formed in cultures of human fetal liver fragments (fetus at 10 weeks). (B) Selected ion monitoring (isothermal at 260°) of the TMS ethyl acetate extract of Pr metabolites formed in cultures of isolated human fetal liver cells (fetus at 24 weeks).

The first GC method for the quantitation of other benzodiazepines also relied upon this acid hydrolysis step but has now been superseded by the GC separation of the intact benzodiazepines. The use of the electron capture detector for increased sensitivity and selectivity in the detection of both the intact benzodiazepines and their metabolites or the acid hydrolysis products is also well documented [9, 10]. However, to our knowledge, there is only

one example of the use of a GC—MS system operated in the selected ion monitoring mode for the analysis of the benzodiazepines pivoxazepam and 2'chloropivoxazepam [13]. The greater specifity which this method can afford is, however, vitiated since analysis follows acid hydrolysis to the benzophenone, where both drug and major metabolites result in the same product.

We found that Pr and its metabolites which have been identified in the human [11] (Scheme I) can be separated using a well conditioned SP-2250 or OV-17 column (Fig. 5) and quantitated with high sensitivity by an electron capture detector.

Scheme I



Although Pr-OH elutes much later than the other compounds from this column, it could also be quantitated, although with reduced sensitivity. Therefore, if low concentrations of Pr-OH (<50 ng/g) were to be determined, the samples were trimethylsilylated and then injected again (Fig. 6). The minimum-detectable quantities of all compounds studied in the injected samples were in the low pg-range (Table II). Blood serum containing 5 ng/ml and fetal tissue containing 5 ng/g of Pr and metabolites could be quantitated by our method with signal-to-noise ratios of >10 on the electron capture detector using 50—200 μl (mg) sample sizes. Lower concentrations (1 ng/mg and 1 ng/g, respectively) could be analyzed if 1 ml or 1 g biological material, respectively, were available.

Selected ion monitoring proved to be of comparable sensitivity for the quantitation of the benzodiazepines studied. We found that the selectivity of the technique for the determination of this class of compounds exceeded that of electron capture GC. Analysis of the trimethylsilylated sample was

sufficient to quantitate all Pr metabolites in a single experiment.

The day to day variation of our method is acceptable as expressed by the relative standard deviations in Table II. Although oxazepam (if it is not trimethylsilylated prior to injection) apparently rearranges to the corresponding quinazolin carboxaldehyde [14, 15], it could be quantitated with slightly higher standard deviations by electron capture GC (Table II). Following trimethylsilylation, the intact molecule with a molecular weight of 429 could be detected (Fig. 7B).

Much lower relative standard deviations result for samples analyzed on the same day. We routinely processed three spiked serum samples (containing between 4 and 40 ng of Ox, DD, Pr, PrOH per 200 µl) with 10—15 serum and fetal tissue samples per day.

Of the three extraction methods which were compared, we now routinely use method (a), the single step ethyl acetate extraction. Method (b) with the Extrelut columns is comparable with (a) regarding speed and ease, and led to higher recoveries of Pr-OH while all other compounds studied had poorer yields. Work is in progress to evaluate possible techniques to increase further the efficiency on the small Extrelut columns. Prepurification of the Extrelut may also result in more acceptable baselines necessary for high sensitivity work. Method (c) is faster and more convenient than (a) and (b) but not as sensitive; the limit of sensitivity was, for Pr and DD, 20—30 ng/ml which is in accordance with the findings of Rutherford [8] who used method (c) for the isolation of diazepam metabolites.

We have applied our method during the past year to the study of the diaplacental passage of Pr in early human pregnancy (Fig. 8) [16]. Fetal tissue

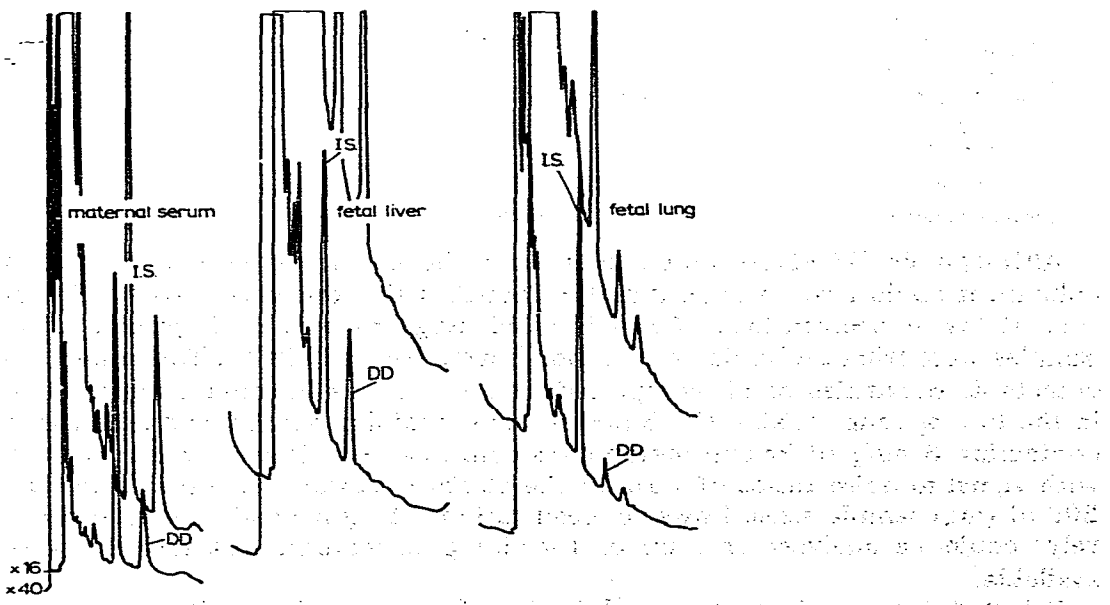


Fig. 8. Electron capture gas chromatograms of ethyl acetate extracts (extraction method, a) of maternal serum, human fetal liver and human fetal lung. The patient received 10 mg Pr 25 h 50 min prior to interruption of pregnancy (17 weeks gestation). DD concentrations were: maternal serum, 74 ng/ml; fetal liver, 151 ng/g; fetal lung, 39 ng/g.

samples were homogenized and extracted in the same manner as serum and plasma samples. Our results indicate that DD, the main metabolite of Pr, accumulates in the fetal liver (Fig. 8). In placenta and fetal heart, concentrations of DD were found which were comparable to the corresponding maternal blood levels, while the other fetal tissues contained much lower concentrations. Accumulation of DD in the fetal liver had previously been detected following maternal diazepam intake [17].

The methods presented in this paper have also been applied extensively to the study of Pr metabolism in human fetal liver in vitro. Both in liver organ cultures (Fig. 6) and cultures of isolated human fetal liver cells (Fig. 7) extensive metabolism of Pr was found. The main metabolites were DD and Pr-OH which were present in comparable amounts, and Ox was also detected (Fig. 7B), albeit in lower concentrations [6].

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