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Drug plasma binding and non-esterified fatty acids: methodologic considerations

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Summary

The in vitro increase in plasma levels of non-esterified fatty acids (NEFA) has been investigated in non-heparinized subject groups of differing lipoprotein lipase activity and degree of lipaemia. The influence of standard blood and plasma collection and equilibrium dialysis techniques on the extent of lipolysis has been assessed. Marked increases in plasma NEFA level occurred during equilibrium dialysis. Respective mean increases of 85% and 230% were observed in plasma collected from non-pregnant (fasted and non-fasted) and parturient subjects after dialysis at 37°C for 26 h. On the in vitro addition of ammonium heparin to blood, no difference ($P > 0.05$) in plasma NEFA was detected between heparin concentrations of 12.5 IU/ml and 41.7 IU/ml. In pregnant and non-pregnant subjects, no alteration ($P > 0.05$) in plasma NEFA levels was noted on storage of blood and plasma in ice or at room temperature for periods of up to 2 h. Spurious binding estimates, however, may be a direct result of in vitro lipolysis for those drugs displaced from binding sites by NEFA and/or avidly bound to lipoproteins. Perturbation to the human serum albumin binding of diazepam (400 ng/ml) and ibuprofen (20 μg/ml) has been demonstrated at NEFA concentrations spanning those induced in vitro during equilibrium dialysis. The results of this study may also explain several anomalies and discrepancies in reported plasma binding estimates.

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Introduction

Lipolysis, when occurring in vitro, will have obvious important consequences in drug plasma binding studies since it may reduce plasma lipoprotein concentration and liberate non-esterified fatty acids (NEFA), potent modifiers of the binding of many drugs and endogenous compounds (Rudman et al., 1971; Curzon et al., 1973; Fredholm et al., 1975; Tsutsumi et al., 1975). Recently lipolysis has been implicated in certain blood and plasma collection and plasma binding studies. The collection of blood via a 'heparin-lock' may produce a transient increase in plasma NEFA level (Fraser et al., 1961) and has been associated with decreased plasma binding of propranolol (Wood et al., 1979) and diazepam (Desmond et al., 1980). More recent evidence, however, suggests that the reported increases in plasma NEFA level after heparin administration may be largely attributed to in vitro formation by lipolysis (Giacomini et al., 1981). It appears, moreover, that equilibrium dialysis may be subject to interference from in vitro lipolysis (Ridd et al., 1980). Increase in plasma NEFA of greater than 100% have been reported after equilibrium dialysis at 20°C for 18 h (Nilsen et al., 1977). In other work, alterations in plasma lipoproteins occurring during equilibrium dialysis have been postulated to account for the low estimate of plasma binding of demethylchlorimipramine (Bertilsson et al., 1979).

The aim of the present work was to investigate the nature and magnitude of alterations in plasma NEFA level associated with established blood and plasma collection and handling techniques and in plasma binding determination by equilibrium dialysis. Subject groups (non-heparinized) with differing lipoprotein lipase activity and degree of lipaemia (Van Duyne and Havel, 1960) were considered.

Materials and methods

Subjects

Thirty healthy subjects, aged between 21 and 40 years, none of whom were obese or showed any evidence of disease, comprised two subject groups. Group I consisted of 3 males and 13 non-pregnant females. Group II consisted of 14 pregnant women either being electively induced into labour at term with synthetic oxytocin or undergoing spontaneous labour and delivery.

Blood collection

Blood was collected by venipuncture of an antecubital vein, transferred to heparinized plastic tubes (125 IU ammonium heparin, Disposable Products, Sydney, Australia), mixed and plasma separated by centrifugation at 1000 *g*. This practice was standard but storage and handling procedures were varied, depending on the nature of the desired investigation.

In vitro heparin

Blood was collected from 4 Group I subjects, 5 h after a light breakfast, and from 2 Group I subjects after a light meal. Blood was collected also from one Group II

subject on admission to hospital and from one Group II subject at delivery. For all subjects, 2 replicate blood aliquots of 3 ml and of 10 ml were immediately transferred to 4 separate heparinized tubes (125 IU ammonium heparin), thereby achieving approximate heparin concentrations of 41.7 IU/ml and 12.5 IU/ml, respectively. Plasma was separated immediately at room temperature and total plasma NEFA concentration was determined in duplicate for each sample.

Temperature and time studies

Blood was collected from 4 Group I women, 3 h after a light breakfast, and from 4 Group II subjects during labour. After storage of blood and plasma at room temperature (25°C) or in ice for various periods of time as outlined in Fig. 1, total plasma NEFA was determined.

Freezing and equilibrium dialysis

Equilibrium dialysis was performed by dialyzing plasma (5 ml) against Sorensen's phosphate buffer (5 ml, 0.067 M, pH 7.4) at 37°C in glass dialysis half-cells joined by a Visking membrane.

Blood was collected from 2 Group II subjects on admission to hospital (day prior to induction of labour), during labour, at delivery, and on the first, second and fifth days postpartum. Plasma was separated at room temperature and total NEFA level determined immediately. After storage at −18°C to −22°C for one week, plasma

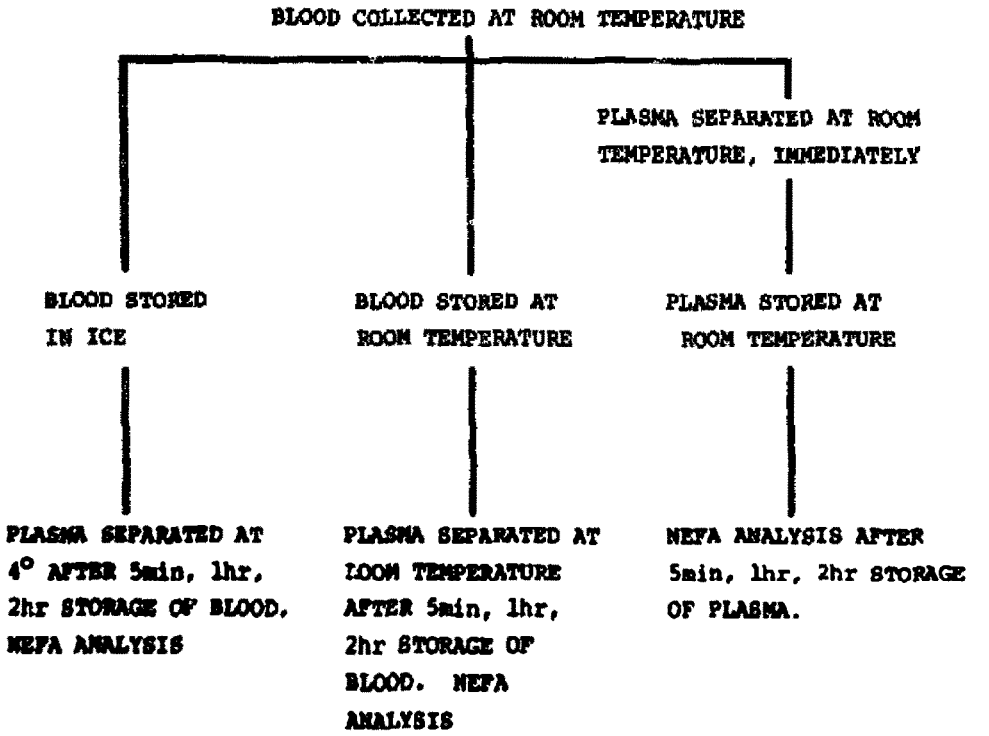

Fig. 1. Time and temperature conditions for the collection of blood, storage of blood and plasma, and analysis of NEFA.

was thawed at room temperature and total NEFA level determined again prior to equilibrium dialysis. After 26 h of dialysis, total NEFA was quantitated by analysis of aliquots from the plasma and buffer compartments.

Blood was collected from 3 Group I subjects after a 12 h fast and from 3 Group I subjects after a light meal. Blood was similarly collected from 3 Group II subjects on admisstion to hospital and from 3 Group II subjects at delivery. In all cases, plasma was separated immediately at 4°C and dialyzed at 37°C for periods up to 26 h. Total NEFA was determined at various time points during the dialysis period, a different dialysis cell being sampled in each case.

NEFA and human serum albumin binding

Palmitic and oleic acids (Sigma Chemicals, U.S.A.) were introduced in equimolar concentrations to a 3.5% human serum albumin (HSA; Behringwerke, Marburg) buffer solution using a Celite addition technique (Celite 545, Johns Manville; Spector and Hoak, 1969). A final total NEFA concentration of 2800 μM was obtained. The binding of [^{14}C]diazepam (400 ng/ml) (Roche Products, Basle) was determined, by liquid scintillation counting (Ridd et al., 1981), in this solution and after serial dilution with blank Celite-treated 3.5% HSA buffer solutions to achieve final NEFA concentrations of 2140 μM, 1800 M, 1140 M, 740 M and 245 μM. The binding of [^{14}C]ibuprofen (20 μg/ml) (Boots Chemicals, U.K.) was similarly determined in these solutions. Albumin concentration was unaltered by Celite treatment and no alteration in albumin and total NEFA concentrations occurred as a result of dialysis.

Analysis of NEFA

Concentrations of NEFA in plasma, HSA and buffer solutions were determined by gas chromatography. To plasma (0.25 ml), HSA solutions (0.25 ml) or buffer (1.0 ml) were added hydrochloric acid (0.5 ml, 5 N) and internal standard (20 μg heptadecanoic acid in 1.0 ml dichloromethane). The mixture was extracted with dichloromethane (4.0 ml) by vortexing for 1 min and centrifuging at 1000 *g* for 10 min. The organic phase was transferred to a glass evaporation tube and concentrated to approximately 10 μl on a water bath at 48°C. Free carboxyl groups were derivitized by adding 10 μl Methyl-8 (Pierce Chemicals, IL) to the tube which was then vortexed for 10 s. A 5 μl aliquot of the extract was injected into a Hewlett-Packard 5730A series gas chromatograph equipped with a flame ionization detector. Resolution was achieved on a glass column, 2 m × 3 mm i.d., packed with 3% OV-1 on Gas Chrom (100/120 mesh) and maintained at 195°C. Inlet and detector temperatures were 250°C and gas flow rates were nitrogen 30 ml/min, hydrogen 33 ml/min and air 300 ml/min. All peaks of interest eluted within 15 min. Quantitation of the major fatty acids present in human plasma, that is, myristic, palmitic, oleic, linoleic, stearic and arachidonic acids, was achieved and their individual concentrations summed to give an estimate of total NEFA (n = 6, CV = 4%, mean concentration 435 μM). Linear calibration plots were obtained over the plasma NEFA concentration range 37–2400 μM.

Statistical analyses

The Wilcoxon signed-ranks test (Colquhoun, 1971) was used to asses the significance of changes in plasma NEFA concentration.

Results

In vitro heparin

For pregnant and non-pregnant subjects, no significant difference ($P > 0.05$) in plasma NEFA could be demonstrated between samples containing 12.5 IU/ml and 41.7 IU/ml heparin (Table 1).

Temperature and time studies

The total levels of plasma NEFA measured after storage of blood or plasma at room temperature or in ice for various periods of time are presented in Table 2. Fluctuations in plasma NEFA level occurred in both pregnant and non-pregnant subjects and approached method reproducibility limits. The levels of plasma NEFA were generally higher in pregnant than in non-pregnant subjects. In both subject groups, no sigifnicant difference in plasma NEFA level was observed after 2 h storage of blood at room temperature ($P > 0.05$), blood in ice ($P > 0.05$) or plasma at room temperature ($P > 0.05$).

Freezing and equilibrium dialysis

Plasma NEFA levels were determined in 2 Group II subjects: immediately on separation of plasma, after blood collection at room temperature; on thawing, after storage of plasma at −18°C to −22°C; and after equilibrium dialysis for 26 h (Fig. 2). On collection, plasma NEFA levels were within the range 61–747 μM and

TABLE I

INFLUENCE OF IN VITRO HEPARIN CONCENTRATION ON PLASMA NEFA LEVEL

Subject		Plasma NEFA μM (mean ± S.D.) *	
Group I		12.5 IU/ml heparin **	41.7 IU/ml heparin **
1	Fasting	694 ± 17	702 ± 23
2	Fasting	500 ± 26	489 ± 12
3	Fasting	862 ± 17	826 ± 10
4	Fasting	634 ± 39	621 ± 21
5	Non-fasting	280 ± 37	259 ± 34
6	Non-fasting	275 ± 16	289 ± 18
Group II			
1	Admission	650 ± 14	687 ± 22
2	Delivery	885 ± 12	908 ± 13

* Data are based on duplicate analyses of 2 replicate plasma samples.

** No significance difference could be demonstrated in plasma NEFA between 12.5 and 41.7 IU/ml heparin ($P > 0.05$, Wilcoxon signed ranks test).

TABLE 2

THE INFLUENCE OF TIME AND TEMPERATURE OF STORAGE OF BLOOD AND PLASMA ON TOTAL PLASMA NEFA (μM)

Subject	Blood room temperature			Blood in ice			Plasma room temperature		
	0 h	1 h	2 h	0 h	1 h	2 h	0 h	1 h	2 h
Group I									
1	295	294	325	290	265	290	295	265	297
2	208	204	221	210	190	192	208	216	225
3	265	275	258	264	265	270	265	308	285
4	378	398	401	375	320	320	378	359	358
Group II									
1	383	–	429	384	352	358	383	390	354
2	353	378	398	361	349	343	353	322	380
3	506	501	478	506	480	510	506	500	507
4	482	510	496	498	462	478	482	479	462

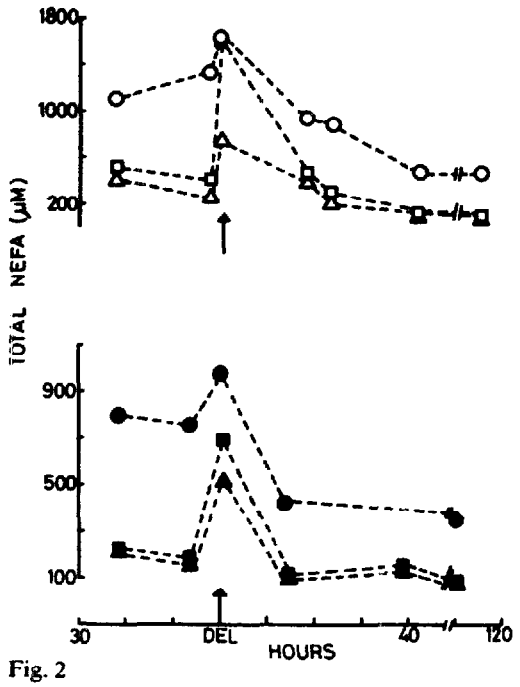

Fig. 2

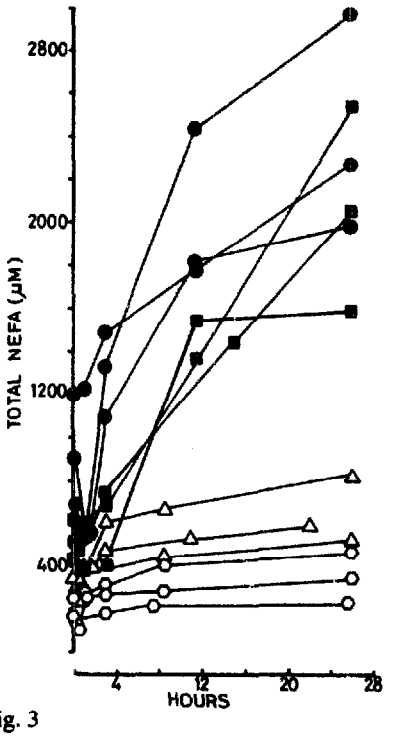

Fig. 3

Fig. 2. Plasma NEFA level-time profiles observed in 2 Group II women: immediately on separation of plasma, after blood collection at room temperature (△, ▲); on thawing, after storage of plasma at −18°C to −22°C (□, ■); and after equilibrium dialysis for 26 h (○, ●). Delivery is indicated by an arrow.

Fig. 3. Plasma NEFA concentrations observed during equilibrium dialysis for Group II subjects on admission to hospital (■) and at delivery (●); and for fasting (△) and non-fasting (○) Group I subjects.

in both subjects the plasma NEFA level–time profiles were qualitatively similar, demonstrating a higher concentration at delivery and a lower concentration on the fifth day postpartum when compared with plasma NEFA levels determined on admission to hospital. On thawing, an average of 15% increase in plasma NEFA level was observed, compared with collection levels, with delivery samples displaying the most prominent rise. Plasma NEFA levels were within the range 87–1600 μM and paralleled the plasma NEFA level–time profile obtained on collection. After equilibrium dialysis, plasma NEFA concentrations (range 346–1625 μM) were markedly enhanced and, compared with predialysis values, increases of 300% and 250% were displayed in predelivery and postdelivery samples, respectively. Again, the observed plasma NEFA level–time profile mimicked that shown on collection and after thawing.

Initially, plasma NEFA levels were higher in pregnant subjects on admission to hospital (mean 546 μM) and at delivery (mean 909 μM) than in non-pregnant subjects in fasting (mean 348 μM) and non-fasting (mean 190 μM) states (Fig. 3). For plasma collected under all conditions, fluctuations in NEFA levels were observed during the early periods of dialysis. In both subject groups, increases in plasma NEFA were significant by 3 h ($P < 0.05$). On completion of dialysis an approximate 85% increase was noted in fasting (mean 642 μM) and non-fasting (mean 345 μM) Group I subjects. Much greater mean increases of 282% and 178% were observed in pregnant subjects on admission (mean 2059 μM) and at delivery (mean 2401 μM), respectively.

NEFA and human serum albumin binding

Diazepam % free and ibuprofen % free, determined in Celite-treated 3.5% HSA buffer solutions at the respective concentrations of 400 ng/ml and 20 μg/ml, increased non-linearly with increasing concentrations of palmitic and oleic acids, spanning the range in total NEFA level observed during equilibrium dialysis (Fig. 4).

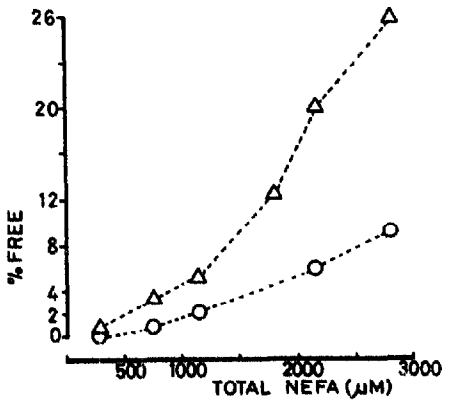

Fig. 4. Variations in diazepam % free (△) and ibuprofen % free (○) in 3.5% HSA solutions with increasing NEFA concentrations in vitro. Total NEFA concentrations span those observed in vitro during dialysis of plasma from parturient and non-pregnant subjects. Binding was determined at the respective diazepam and ibuprofen concentrations of 400 ng/ml and 20 μg/ml.

Discussion

Lipolysis, occurring during blood and plasma collection and plasma binding determination, may provide an important, generally unrecognized, source of spurious estimates in drug binding studies and perhaps plasma concentration data. An enhancement of plasma NEFA level, resulting from in vitro lipolysis, may markedly increase or decrease the albumin binding of many drugs (Birkett et al., 1977). Alternatively, for drugs largely bound to lipoproteins, lipolysis may reduce the concentration of binding substrate and thus artificially lower estimates of plasma binding. The putative in vitro lipolysis associated with standard blood and plasma collection and equilibrium dialysis techniques has been investigated in the present study.

Blood is usually collected into tubes containing a fixed amount of heparin. The final plasma heparin concentration varies, therefore, depending on the volume of collected blood. In the present study, no significant difference ($P > 0.05$) in plasma NEFA level was detected between the in vitro heparin concentrations 12.5 and 41.7 IU/ml (Table 1). A previous report indicated that heparin, in vitro, may inhibit lipoprotein lipase activity (Elkeles, 1974). However, the present findings are consistent with the majority of studies which show that plasma binding of diazepam (Routledge et al., 1980) propranolol (Wiegand et al., 1980; Wood et al., 1979) and bilirubin, diphenylhydantoin, salicylic acid and warfarin (Wiegand et al., 1980) is unaltered by the addition of heparin in vitro. In one report, however, reduced plasma binding of quinidine was linked with in vitro heparin (Kessler et al., 1979).

Prior to analysis, plasma is generally stored at 4°C or frozen at −20°C. Some delay between collection of blood and separation of plasma is frequently unavoidable and blood or plasma may stand at room temperature or in ice for variable periods. Sampson and Hensley (1975) reported that NEFA level may increase in blood or plasma stored at room temperature or in ice for 1 h. In the present study, no significant or consistent alteration was noted in plasma NEFA levels ($P > 0.05$) on storage of blood or plasma under these conditions for periods of up to 2 h (Table 2). Furthermore, no appreciable change in plasma NEFA concentration had occurred after 2 h at the harsher conditions of equilibrium dialysis, namely agitation at 37°C (Fig. 3). A similar inconsistent but variable alteration in NEFA level with time and temperature of storage has been demonstration in plasma from non-pregnant non-heparinized subjects (Giacomini et al., 1981).

Equilibrium dialysis is the most extensively used procedure for estimating plasma binding and is generally considered to be the most reliable. In the present investigation, marked increases in NEFA levels were observed after equilibrium dialysis at 37°C for 26 h in plasma collected from both parturient and non-pregnant subjects (Figs. 2 and 3). The albumin binding of diazepam (400 ng/ml) and ibuprofen (20 μg/ml) was shown to be profoundly decreased by oleic and palmitic acid concentrations analogous with the range of plasma NEFA concentrations observed during dialysis (Fig. 4). Such NEFA increase may explain the discrepant estimates of plasma binding obtained with equilibrium dialysis compared with alternative methods, for identical plasma samples or similar patient groups. Lipolysis during dialysis

will perturb the binding of NEFA-sensitive drugs and/or those bound largely to lipoproteins, yielding artificially low estimates relative to binding methods which avoid in vitro lipolysis. Using equilibrium dialysis, the plasma binding of quinidine (Kessler et al., 1979; Fremstad et al., 1976), diphenylhydantoin (Kurata and Wilkinson, 1974) demethylchlorimipramine (Bertilsson et al., 1979) and sulphaguanidine and thiopentone (Kurz et al., 1977) was lower than with other methods.

It has been reported that the binding of diphenylhydantoin and warfarin (Gugler et al., 1974), determined by equilibrium dialysis, was reduced in plasma at a NEFA:albumin molar ratio of 1.5 whilst ratios of 2 and 3 were required in albumin solutions to achieve a similar reduction. However, since plasma samples contain a 'triglyceride reservoir', they may be subject to lipolysis during equilibrium dialysis whilst NEFA–albumin solutions will remain unaffected. Thus estimates of plasma NEFA concentration determined *pre*-dialysis may be considerably lower than those existing in the plasma *post*-dialysis.

The present findings indicated that lipolysis commonly occurs during equilibrium dialysis. However, the artefact appears to vary in magnitude between subject groups. Increases in plasma NEFA observed during dialysis of plasma from parturients (mean 230%; Figs. 2 and 3) were markedly greater than the maximum increase (85%) shown with non-pregnant subjects in fasting and non-fasting states (Fig. 3). Marked interindividual variability in dialysis-enhanced plasma NEFA has been reported in young healthy volunteers (Nilsen et al., 1977). Differences in subject lipaemia may account for intersubject variability in equilibrium dialysis binding estimates for a given drug in similar (Glassman et al., 1973; Piafsky and Borgå, 1977) or differing, patient groups.

No marked change in NEFA level was observed during the first 2 h of dialysis (Fig. 3). Procedures employing short dialysis time or rapid separative techniques such as ultrafiltration should eliminate the lipolysis artefact and provide improved alternatives in plasma binding methodologies.

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