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Determination of amino acid tissue concentrations by microdialysis: method evaluation and relation to plasma values

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Summary. Microdialysis is an in vivo technique to monitor tissue concentrations of low molecular weight substances by means of a continuously perfused artificial capillary with a semipermeable membrane placed into the region of interest. The suitability of microdialysis to determine tissue concentrations of amino acids was evaluated in vitro by placing the catheter into Ringer buffer or into a plasma protein (50 g/l) solution containing 32 different amino acids (150 μmol/l each). All amino acids tested crossed freely the microdialysis membrane with recoveries close to 100%. Microdialysis fluid was sampled from subcutaneous tissue of five newborns and amino acid content analysed. Total and non protein bound amino acids were determined in the patients plasma by acid precipitation or ultrafiltration, respectively. Mean subcutaneous tissue concentrations were lower as compared to plasma for taurine, serine, alanine, aspartate, glutamate and ornithine and higher for valine, isoleucine, leucine, methionine, phenylalanine, tyrosine and arginine, indicating net uptake or release of amino acids from subcutaneous tissue. Thus, microdialysis offers a convenient and minimal invasive way to study tissue amino acid composition and appears to be a promising analytical tool for the study of amino acid metabolism in vivo.

Keywords: Amino acids – Microdialysis – Ultrafiltration – Protein binding – Subcutaneous tissue – Newborn

Introduction

Microdialysis is a recently established technique for the continuos monitoring of low molecular weight substances in the extracellular fluid of various tissues. The principle is based on an artificial capillary built of a semipermeable membrane with a molecular exclusion size of 20 kD. The catheter with the capillary on its tip is placed into the tissue of interest and continuously

perfused. Thus, analytes with a molecular weight below the exclusion size of the catheter are expected to cross the membrane leading to an equilibrium between the extracellular fluid concentration of the (free) analyte and its concentration in the microdialysis fluid (Ungerstedt, 1991; Hillered and Persson, 1999a).

In humans, tissues studied so far include brain (Hamani et al., 1997; Maggs et al., 1997), bone (Thorsen et al., 1996), muscle (Maggs et al., 1997; Lonnroth, 1997), myocard (Habicht et al., 1998; Kennergren et al., 1999) and subcutaneous or adipose tissue (Lonnroth, 1997). In addition, in animal experiments the feasibility was also demonstrated for spinal cord, pituitary gland, eye, kidney, liver, pancreas, intestine, spleen, lung and blood (Elmquist and Sawchuk, 1997). Thus, microdialysis has proved to be a potent and promising tool for the study of tissue metabolism. In clinical practice, microdialysis has been established for bedside monitoring of glucose, lactate, pyruvate, glycerol and urea especially in the neurointensive care (Persson and Hillered, 1992; Hillerd and Persson, 1999b).

Apart from proteins and peptides, amino acids in plasma as well as in the interstitial fluid may occur either free or non-covalently bound to plasma proteins. Only for their free fraction an equilibrium with the perfusion fluid of the microdialysis system is expected. Thus, amino acids measured in microdialysis fluid will reflect the amount of free amino acids in the surrounding space. There are only few informations available on protein binding of amino acids in plasma or tissue fluids (Perry and Hansen, 1969). Nevertheless, it is generally believed that amino acids (except of tryptophane) occur predominantly in their free form. Using microdialysis, numerous studies on the concentrations of the neuroexcitotoxic amino acids glutamate and aspartate in brain interstitial fluid had been performed (Persson and Hillered, 1992; Hillerd and Persson, 1999b). Recently, selected other amino acids had also been studies in brain (Shah et al., 1999), muscle (Kennergren et al., 1999; Gutierrez et al., 1999) and adipose tissue (Frayn et al., 1991; Dabrosin et al., 1997). However, no systematic evaluation had been conducted to evaluate the feasibility of microdialysis for the measurement of amino acid tissue concentrations. Moreover, there is only sparse information available on amino acid levels in subcutaneous tissue in humans (Frayn et al., 1991; Dabrosin et al., 1997) and their relation to plasma levels.

In the present study we set out to investigate the usefulness of microdialysis to determine tissue concentrations of amino acids. In a preliminary attempt we studied subcutaneous tissue and plasma amino acid concentrations in newborn children.

Materials and methods

Patients

Five newborn children were enrolled in a study for continuos bedside monitoring for hypoglycaemia and lactic acidemia by microdialysis in the setting of a neonatal intensive care unit. The parents gave written informed consent to the investigations and the study protocol was approved by the local ethical committee. One of the children was preterm

and small for gestational age, the others full-term. All had documented repeated hypoglycaemia before enrolment. Gestational age ranged from 28,1 to 37 weeks. Microdialysis was started at a patient age from 0,3 to 5,7 weeks and a patient weight from 1,240 to 2,840 g, respectively. Microdialysis duration ranged from 8 to 16 days.

Microdialysis and sample collection

We used a CMA 70 microdialysis catheter (CMA/Microdialysis, Solna, Sweden) with a membrane length of 10 mm and a diameter of 0.6 mm. The molecular exclusion size of the polyamide membrane was 20 kD. After local anaesthesia with a lidocain containing ointment (EMLA Creme, Astra, Wedel, Germany) the catheter was placed through a 18 g canula into the subcutaneous tissue of the right or left lateral upper leg under sterile conditions. In one patient a second catheter was placed into the contralateral leg before the first one was removed. In this patient samples from both catheters were collected in parallel. The catheter was perfused by a CMA 107 microdialysis pump (CMA/Microdialysis, Solna, Sweden) at a perfusion rate of 0.3 μl/min with Ringer solution. Perfusion was performed for 3 hours to gather a 50 μl sample. Samples were collected into specially capped microvials (CMA/Microdialysis, Solna, Sweden) to avoid evaporation. The collection period was timed in accordance to a regular venipuncture performed for routine laboratory evaluation. At this occasion, blood for amino acid determination (approximately 300 μl) was collected into EDTA containing tubes (Sarstedt, Nümbrecht, Germany) and centrifuged within half an hour. Plasma was used for amino acid determination and ultrafiltrate preparation without prior freezing.

Evaluation of the microdialysis catheter

Partition equilibrium of the catheter for free amino acids was studied in two ways. First, Benson Calibration Standard (Eppendorf, Hamburg, Germany) was supplemented with aqueous solutions of asparagine and glutamine (Sigma, Deisenhofen, Germany) and diluted to a final concentration of 150 μmol/l for each amino acid with Ringer solution supplemented with 0,55 μmol/l NaCl to account for the dilution by the aqueous amino acid solutions. The final pH was 7.2. Second, the recovery of amino acids from the catheter out of a protein containing matrix was studied. For this purpose, Benson Calibration Standard was supplemented with asparagine and glutamine and diluted to a final concentration of 150 μmol/l for each amino acid with a plasma protein solution containing 31 g/l albumin and 10 g/l immunoglobulins (Biseko, Biotest, Dreieich, Germany) supplemented with 0,55 μmol/l NaCl. The catheter was immersed into a 3.6 ml aliquot of the respective solutions (kept at 37°C and stirred to account for possible concentration gradients). Microdialysis was performed at the same flow rate as in vivo (0.3 μl). After an equilibration time of 6 hours the effluent was collected for 3 hours to yield an aliquot of 50 μl. Both experiments were performed with three different catheters. Prior to each microdialysis experiment an aliquot of the solution was taken for amino acid analysis.

Preparation of ultrafiltrate

To test the suitability of ultrafiltration for the determination of free amino acids 100 μl aliquots of the standard solutions described above were applied to Microcon 10 Microconcentrators (Amicon, Witten, Germany) with a molecular exclusion size of the membrane of 10 kD. The device was centrifuged in an fixed angle rotor centrifuge at 2,500 g for 10 min at room temperature and the ultrafiltrate subjected to amino acid analysis.

The concentrations of free amino acids in plasma were determined by loading 50 to 100 μl of plasma onto the Microcon 10 Microconcentrator and centrifugation in a fixed

angle rotor centrifuge at 5,000 g for 10 min at room temperature. Again, the resulting protein-free ultrafiltrate was used for analysis.

Amino acid determination

Amino acids were separated by ion exchange chromatography using a five step gradient program for physiological fluids and detected after ninhydrin reaction at 570 nm on a Eppendorf Biotronik LC 3000 analyser (Eppendorf, Hamburg, Germany). Quantitation was performed by an external amino acid standard (Benson Calibration Standard, Eppendorf, Hamburg, Germany) supplemented with asparagine and glutamine (Sigma, Deisenhofen, Germany) at a final concentration of 200 μmol/l for each amino acid. Data were recorded and processed using the chromatography software Gynkosoft (Gynkotek, Germering, Germany). Plasma (80 μl) was deproteinized by the addition of 20 μl sulfosalicylic acid (10%, w/v), centrifuged (10.000 g, 10 min, 4°C) and the supernatant diluted with an equal volume of a sample diluent buffer to adjust the sample pH. All microdialysis fluids or ultrafiltrates were diluted with an equal volume of sample diluent without prior deproteinisation.

Statistical analysis

Statistical calculations were performed using the SPSS software package (SPSS/PC 7.0, Chicago, USA). Tests for statistical significance were performed using the Students-t-test.

Results

All amino acids tested crossed freely the 20 kD polyamide membrane of the CMA microdialysis catheter. In the in vitro experiment in an aqueous solution the concentrations of amino acids were slightly higher in the catheter effluent as compared to the immersion fluid ($p < 0{,}05$) and recoveries ranged from 102 to 118% of total (Table 1). When the catheter was placed into blank Ringer solution no amino acids at all ($<2 - 5$ μmol/l) were found in the catheter effluent (data not shown). In these experiments, we observed a net efflux of water from the microdialysis catheter to the immersion fluid after longer dialysis time accounting for a dilution of 5.9% within 24 hours.

When tested with the amino acids diluted with plasma protein solution, again, free diffusion could be seen for all amino acids with recoveries ranging from 91 to 104% of total (Table 1). Marked differences were only seen for carnosine, histidine and 3-aminopropionic acid (25.5, 164.1 and 164.2% of total, respectively). Except for these compounds, again, the amino acid concentrations in the catheter effluent were slightly higher as compared to the free fraction in the surrounding compartment (not significant) as shown in Table 1.

When investigating the ultrafiltration devices with the aqueous standard solution no significant differences could be observed for amino acid concentrations before and after filtration with recoveries ranging from 83 to 108% (Table 1). When blank buffer was filtered through the device, no amino acids at all ($<2 - 5$ μmol/l) were found in the ultrafiltrate (data not shown). When investigated with amino acids diluted in plasma, only a small fraction

Table 1. Ultrafiltrate and microdialysis fluid recoveries as % of total tested in vitro

	Standard (150 μmol/l) diluted in Ringer solution		Standard (150 μmol/l) diluted in protein solution	
	Ultrafiltration (Mean ± SD)	Microdialysis (Mean ± SD)	Ultrafiltration (Mean ± SD)	Microdialysis (Mean ± SD)
Phosphoserine	97.9 ± 0.4	109.0 ± 0.2	98.5 ± 0.8	97.9 ± 2.2
Taurine	101.4 ± 0.5	108.1 ± 2.8	97.2 ± 1.1	99.9 ± 1.9
Phosphoethanolamine	98.4 ± 1.5	106.9 ± 4.8	95.6 ± 2.8	99.2 ± 1.3
Aspartic acid	92.6 ± 0.8	107.1 ± 3.9	99.2 ± 1.6	100.5 ± 1.3
Threonine	94.8 ± 2.0	107.2 ± 2.9	97.3 ± 0.9	97.3 ± 0.7
Serine	98.0 ± 1.5	106.6 ± 1.5	96.8 ± 2.5	100.4 ± 0.7
Asparagine	93.7 ± 1.0	105.9 ± 2.4	99.8 ± 1.9	101.5 ± 2.1
Glutamic acid	99.1 ± 1.6	107.9 ± 2.8	96.6 ± 2.6	99.0 ± 2.9
Glutamine	89.5 ± 2.4	118.4 ± 8.9	96.8 ± 2.3	98.4 ± 5.4
2-Aminoadipic acid	93.1 ± 1.7	105.9 ± 4.8	99.6 ± 1.4	99.6 ± 3.4
Glycine	95.3 ± 0.6	107.5 ± 1.7	101.0 ± 3.1	99.4 ± 1.8
Alanine	92.1 ± 1.1	104.5 ± 2.6	97.9 ± 1.5	99.1 ± 1.2
Citrulline	97.1 ± 1.1	105.9 ± 3.8	96.3 ± 4.9	100.2 ± 2.3
2-Aminobutyric acid	95.8 ± 1.0	107.5 ± 3.2	93.3 ± 3.1	98.1 ± 5.4
Valine	104.1 ± 2.6	110.5 ± 2.8	98.8 ± 0.8	98.3 ± 1.3
Cystine	101.0 ± 2.1	107.2 ± 1.8	97.2 ± 0.2	95.4 ± 0.7
Cystathionine	97.4 ± 1.1	107.6 ± 2.7	97.7 ± 0.6	98.6 ± 1.2
Methionine	95.2 ± 0.5	109.7 ± 4.5	96.5 ± 1.4	96.2 ± 2.1
Isoleucine	96.5 ± 1.4	109.5 ± 3.1	96.6 ± 1.1	96.0 ± 0.9
Leucine	95.5 ± 1.0	108.4 ± 4.3	96.5 ± 0.5	100.8 ± 0.4
Tyrosine	95.8 ± 0.7	106.6 ± 2.9	95.9 ± 1.3	97.1 ± 0.8
Phenylalanine	93.4 ± 0.7	107.3 ± 2.7	97.8 ± 0.8	96.5 ± 1.8
2-Aminopropionic acid	108.7 ± 1.2	109.1 ± 4.0	96.9 ± 2.7	164.2 ± 27.1
3-Aminoisobutyric acid	98.2 ± 1.0	102.8 ± 5.5	92.5 ± 3.1	101.7 ± 0.4
4-Aminobutyric acid	94.4 ± 0.8	107.5 ± 3.8	104.7 ± 11.6	99.2 ± 3.9
Histidine	97.7 ± 1.0	106.9 ± 3.9	102.3 ± 8.5	164.1 ± 7.8
3-Methylhistidine	92.5 ± 2.4	109.5 ± 3.3	93.9 ± 2.8	93.6 ± 6.4
l-Methylhistidine	93.2 ± 0.2	109.3 ± 3.9	96.8 ± 0.9	95.5 ± 2.6
Carnosine	84.7 ± 2.1	111.0 ± 9.5	91.5 ± 3.3	25.5 ± 16.8
Ornithine	95.3 ± 1.7	106.5 ± 3.6	96.9 ± 0.4	97.8 ± 3.3
Lysine	88.6 ± 0.7	106.3 ± 6.1	99.3 ± 0.7	97.6 ± 3.9
Arginine	83.2 ± 2.0	106.4 ± 5.6	98.6 ± 0.2	99.4 ± 1.8

was found to be protein bound. In this in vitro system, protein binding on average accounted only for 5% of total for all amino acids tested (Table 1).

Protein binding was also investigated ex vivo in the five patients by ultrafiltration (Table 2). As observed in vitro, the free fraction accounted for the major part of amino acids in plasma. Recoveries were slightly lower than in vitro and ranged from 73 to 100% of total with only a small interindividual coefficient of variation. Details are given in Table 2.

When the concentrations of amino acids in the interstitial fluid of the patients subcutaneous tissue were measured in the microdialysis effluent in the five patients, the pattern of amino acids, grossly, resembled that of plasma.

Table 2. Amino acid concentrations in plasma, ultrafiltrate and microdialysis fluid in the 5 patients

	Plasma	Ultrafiltrat		Microdialysat		
	Median concentration (μmol/l)	Median concentration (μmol/l)	Median ratio to Plasma (%)	Median concentration (μmol/l)	Median ratio to Plasma (%)	Median ratio to Ultrafiltrat (%)
Taurine	74,1	77,9	88,2	48,7	56,1	67,4
Aspartic acid	14,8	15,1	93,2	3,70	29,7	22,1
Threonine	479,8	445,9	92,9	455,9	105,0	101,5
Serine	182,5	169,6	92,9	93,8	85,1	76,2
Asparagine	38,5	47,0	86,2	42,2	109,8	99,4
Glutamic acid	76,2	75,0	99,5	20,0	19,8	21,4
Glutamine	443,5	438,8	96,7	441,3	104,5	100,5
Glycine	286,4	273,0	97,6	225,2	101,8	88,0
Alanine	385,3	401,7	99,5	342,6	87,7	76,6
Citrulline	20,8	22,2	100,2	19,5	93,3	78,7
Valine	150,4	163,7	87,4	169,1	117,2	127,7
Methionine	21,4	20,0	88,5	21,3	111,3	116,0
Isoleucine	45,7	57,2	95,2	70,2	145,3	134,4
Leucine	93,6	83,3	91,4	108,4	125,2	131,3
Tyrosine	38,4	37,9	91,2	47,0	127,0	111,5
Phenylalanine	52,7	42,0	79,7	46,6	108,2	116,4
Histidine	76,8	85,9	89,5	67,8	85,4	90,5
Ornithine	121,7	113,0	92,9	55,0	66,9	63,5
Lysine	201,5	188,5	86,9	180,0	104,2	95,9
Arginine	32,6	25,4	71,8	66,6	219,3	334,6

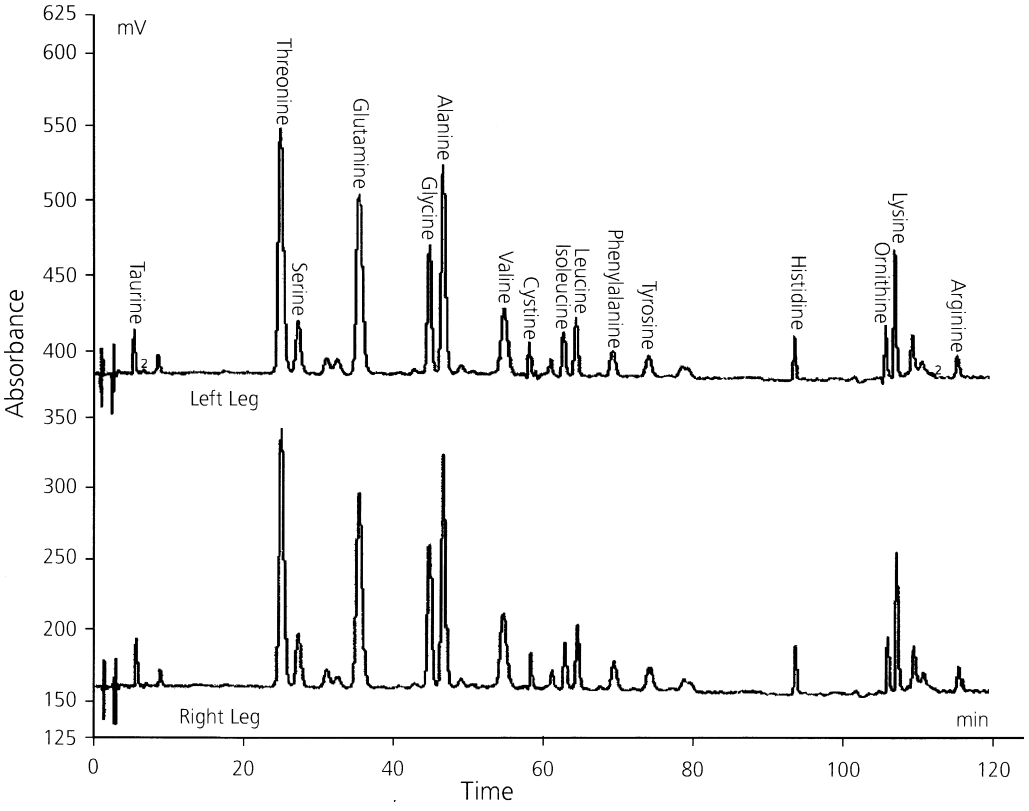

Fig. 1. Ion exchange chromatographic analysis of the subcutaneous tissue amino acid composition from the left (1) and right (2) upper leg collected simultaneously in one patient. Virtually no difference can be seen between both samples

Especially, no amino acids usually not present in plasma were observed in the interstitial fluid. However, there were small but distinct differences in the subcutaneous tissue amino acid composition as compared to total or free amino acids in plasma. Mean values for taurine, serine, alanine, aspartate, glutamate and ornithine were lower than those in plasma (<85% of plasma concentration), whereas the mean concentrations of valine, isoleucine, leucine, methionine, phenylalanine, tyrosine and arginine exceeded those in plasma (>115%). For the remaining amino acids threonine, asparagine, glutamine, glycine, citrulline, histidine and lysine no obvious differences to mean plasma concentrations were observed.

In one patient subcutaneous tissue amino acid concentrations could be measured in parallel from two microdialysis catheters. There was virtually no difference in amino acid concentrations of the subcutaneous tissue from two different locations (Fig. 1).

Finally, insertion and maintenance of the catheter was well tolerated in all five children for up to 16 days. After removal the catheters were tested in vitro for urea equilibrium which was near 100% for all catheters regardless of the time of indwellance (data not shown).

Discussion

In the present study we were able to demonstrate the suitability of microdialysis for the quantitation of 32 major amino acids in the interstitial fluid of tissues. All amino acids studied crossed freely the polyamide membrane of the catheter, regardless of their size, electrical charge or chemical structure. A decreased recovery of carnosine associated with an increase of histidine and 3-aminopropionic acid was found only in the experiment utilizing plasma protein solution as diluent. This might be explained by degradation of carnosine (*N*-(3-aminopropionyl)-*l*-histidine) to 3-aminopropionic acid and histidine most likely by the presence of carnosinase in the plasma protein solution. Thus, the catheter appears to fulfil the necessary prerequisites for its applicability for the determination of tissue concentrations of amino acids.

Assessing the in vitro concentration equilibrium of amino acids across the perfused catheter we found slightly higher concentrations of amino acids in the catheter effluent as compared to the dialysed compartment. This finding might be explained by the fact that the catheter is continuously perfused, leading to a concentration gradient across the length of the catheter with increased influx of the analyte. One other possible explanation might be that the net efflux of water from the catheter in turn results in an increased influx of amino acids. This net water flux might most likely be due to a small accidental difference in the osmolarity of the two buffers used in the experiment. This finding, on the other hand, stresses the very importance of the microdialysis buffer composition. Even small, e.g. disease related changes in the ionic composition of the tissue studied may have impact on the accuracy of the measurement.

The applicability of ultrafiltration for the determination of the free fraction of amino acids in plasma was demonstrated. No binding to or leakage from the ultrafiltration membrane was observed resulting in a nearly complete recovery of all amino acids tested from an aqueous solution. In plasma, amino acids were found to occur mainly in the free form. Only around 5 to 10% of total amino acids were shown to be bound to plasma proteins. This finding is in good agreement with published data (Perry and Hansen, 1969). In the patients plasma we observed on average a somewhat greater extent of protein binding as compared to spiked plasma protein solution. However, it has to be taken into account that the plasma protein solution used in these experiments had a lower total protein content and a different composition as compared to native human plasma.

Although in part reflecting plasma values, amino acid composition of the subcutaneous interstitial fluid revealed a tissue specific pattern. Interestingly, in all patients we found increased concentrations of aromatic and branched chain amino acids as well as of methionine and arginine. This finding suggests synthesis and or release of these compounds by the subcutaneous tissue. On the other hand, decreased concentrations as compared to plasma were found for some neutral amino acids and ornithine indicative for net uptake. In concordance with these findings, uptake of glutamate and aspartate had previously been demonstrated for human (Frayn et al., 1991) as well as for rat

adipose tissue (Kowalski and Watford, 1994; Kowalski et al., 1997). In addition, release of tyrosine had also been observed in adipose tissue of rats (Kowalski and Watford, 1994; Kowalski et al., 1997). In contrast, however, additional uptake of glycine and arginine as well as release of glutamine, serine and taurine had been shown in rats (Frayn et al., 1993). Thus, we describe here a unique pattern of amino acid uptake and release in subcutaneous tissue of newborns that is different as compared to results obtained from adipose tissue. Although fat is an important part of the subcutis in humans, distinct differences could be observed between pure adipose (inguinal) tissue and subcutaneous tissue of the forearm in humans (Frayn et al., 1993). Moreover, tissue composition and metabolic activity of the subcutis in newborns or preterm infants may differ considerable from that in adults. Most interestingly, our data indicate that the subcutaneous tissue in these patients seems not to contribute to net glutamine synthesis. In addition, the homogeneity of the subcutis as a metabolic compartment is also underlined by the finding that tissue concentrations are in very good correspondence if measured in parallel from two limbs.

Thus, microdialysis offers a convenient and minimal invasive way to study tissue amino acid composition. This technique combines the advantage of continuos sampling with the saving of blood volume and may thus represent a promising tool for the facilitated study of amino acid metabolism especially in (preterm) infants. Moreover, minimising or avoidance of blood sampling might also facilitate studies in small laboratory animals. The clinical applicability of microdialysis for the surveillance of nutritional status or treatment as well as its diagnostic value remains to be established in larger trials.

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